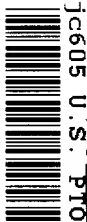


08/05/98



PTO 09/129298

Please type a plus sign (+) inside this box ☒

PTO/SB/05(12/97)

Approved for use through 09/30/00, OMB 0651-0032
Patent and Trademark Office: U.S. DEPARTMENT OF COMMERCE

Under the Paperwork Reduction Act of 1995, no person are required to respond to a collection of information unless it displays a valid OMB control number.

UTILITY PATENT APPLICATION TRANSMITTAL (Only for new nonprovisional application under 37 CFR 1.53(b))	Attorney Docket No.	7991-023-999	Total Pages	49
	First Named Inventor or Application Identifier			
	Charles J. Arntzen			
	Express Mail Label No.	EE 773 773 481 US		

PTO 09/129298



08/05/98

APPLICATION ELEMENTS See MPEP chapter 600 concerning utility patent application contents	ADDRESS TO: Assistant Commissioner for Patents Box Patent Application Washington, DC 20231
<p>1. <input checked="" type="checkbox"/> Fee Transmittal form (Submit an original, and a duplicate for fee processing)</p> <p>2. <input checked="" type="checkbox"/> Specification [Total Pages 49] (Preferred arrangement set forth below) Descriptive title of the Invention Cross Reference to Related Application Statement Regarding Fed sponsored R&D Reference to Microfiche Appendix Background of the Invention Brief Summary of the Invention Brief Description of the Drawings (if filed) Detailed Description Claim(s) Abstract of the Disclosure</p> <p>3. <input type="checkbox"/> Drawing(s) (35USC 113) [Total Sheets <input type="checkbox"/>]</p> <p>4. Oath or Declaration [Total Pages <input type="checkbox"/>] a. <input checked="" type="checkbox"/> Newly executed (original or copy) b. <input type="checkbox"/> Copy from a prior application (37 CFR 1.63(d)) (for continuation/divisional with box 17 completed) [Note box 5 below] i. <input type="checkbox"/> Deletion of Inventor(s) Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).</p> <p>5. <input type="checkbox"/> Incorporation By Reference (useable if Box 4b(is checked) The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.</p>	<p>6. <input type="checkbox"/> Microfiche Computer Program (Appendix)</p> <p>7. Nucleotide and/or Amino Acid Sequence Submission (if applicable, all necessary) a. <input checked="" type="checkbox"/> Computer Readable Copy b. <input checked="" type="checkbox"/> Paper Copy (identical to computer copy) c. <input checked="" type="checkbox"/> Statement verifying identify of above copies</p> <p>ACCOMPANYING APPLICATION PARTS</p> <p>8. <input checked="" type="checkbox"/> Assignment Papers (cover sheet & document(s))</p> <p>9. <input type="checkbox"/> 37 CFR 3.73(b) Statement <input type="checkbox"/> Power of Attorney (When there is an assignee)</p> <p>10. <input type="checkbox"/> English Translation Document (if applicable)</p> <p>11. <input type="checkbox"/> Information Disclosure <input type="checkbox"/> Copies of IDS Statement (IDS)/PTO-1449 Citations</p> <p>12. <input checked="" type="checkbox"/> Preliminary Amendment</p> <p>13. <input checked="" type="checkbox"/> Return Receipt Postcard (MPEP 503) (Should be specifically itemized)</p> <p>14. <input checked="" type="checkbox"/> Small Entity <input type="checkbox"/> Statement filed in prior Statement(s) application, Status still proper and desired</p> <p>15. <input type="checkbox"/> Certified Copy of Priority Document(s) (if foreign priority is claimed)</p> <p>16. <input type="checkbox"/> Other</p>
<p>17. If a CONTINUING APPLICATION, check appropriate box and supply the requisite information: <input type="checkbox"/> Continuation <input type="checkbox"/> Divisional <input type="checkbox"/> Continuation -in-part (CIP) of prior application No: _____</p>	
<p>18. CORRESPONDENCE ADDRESS <input type="checkbox"/> Customer Number or Bar Code Label (Insert Customer No. or Attach bar code label here) Or <input checked="" type="checkbox"/> Correspondence address below</p> <p>NAME <u>Daniel Hansburg</u></p> <p>ADDRESS <u>Kimeragen, Inc., 300 Pheasant Run</u></p> <p>CITY <u>Newtown</u> STATE <u>PA</u> ZIP CODE <u>18940</u></p> <p>COUNTRY <u>United States</u> TELEPHONE <u>215-504-4444</u> FAX <u>215-504-4545</u></p>	

09/129298 PTO 08/05/98

KIMERAGEN, INC.
300 PHEASANT RUN
NEWTOWN, PA 18940
215-504-4444

ATTORNEY DOCKET NO. 7991-023-999

Date: August 5, 1998

Assistant Commissioner for Patents
 Box Patent Application
 Washington, D.C. 20231

Sir:

The following utility patent application is enclosed for filing:

Applicant(s): Charles J. Arntzen, Peter B. Kipp,
 Ramesh Kumar and Gregory D. May

Executed on: C. Arntzen on August 4, 1998
 P. Kipp on August 4, 1998
 R. Kumar on July 31, 1998
 G. May on August 4, 1998

Title of Invention: THE USE OF MIXED DUPLEX OLIGONUCLEOTIDES TO EFFECT LOCALIZED GENETIC
 CHANGES IN PLANTS

Pages of Specification: 48 pages with 72 claims, plus Abstract Sheets of Drawings: None

Prior to calculation of the Application Fee please enter the Preliminary Amendment which is filed of even date.
 An estimate of the application fee due is given below.

PATENT APPLICATION FEE VALUE

TYPE	NO. FILED	LESS	EXTRA	EXTRA RATE	FEE
Total Claims	18	-20	0	\$22.00 each	0.00
Independent	1	-3	0	\$82.00 each	0.00
Minimum Fee					790.00
Multiple Dependency Fee If Applicable (\$250.00)					0.00
Total					0.00
50% Reduction for Independent Inventor, Nonprofit Organization or Small Business Concern (a verified statement as to the applicant's status is attached)					- 395.00
Estimated Filing Fee					\$ 395.00

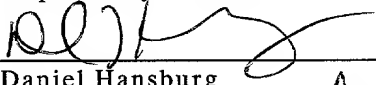
A small entity declaration accompanies this application.

Please charge the required fee to Kimeragen Deposit Account No. 50-0233. A copy of this sheet is enclosed.

This application contains a Sequence Listing in printed and computer readable forms. Applicants hereby state that the contents of the computer readable and printed forms of the Sequence Listing are identical.

Please address all correspondence to the undersigned at the above address.

Respectfully submitted,

 (Reg No. 36,156)
 Daniel Hansburg
 Kimeragen, Inc.
 300 Pheasant Run
 Newtown, PA 18940

Aug. 5, 1998

Enclosure

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

U. S. Application of: Charles J. Arntzen, Peter B. Kipp, Ramesh Kumar and Gregory D. May

Serial No.: To be assigned

Group Art Unit: To be assigned

Filed: Even date herewith

Examiner: To be assigned

For: The Use of Mixed Duplex Oligonucleotides Attorney Docket No.: 7991-023-999
To Effect Localized Genetic Changes in
Plants

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Box Patent Application
Washington, D.C. 20231

Sire:

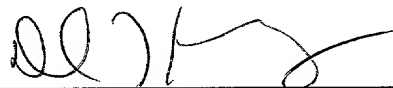
Prior to calculation of the Application Fee of the captioned application please cancel
without prejudice claims 1-53.

REMARKS

Claims 54-72 are pending in the application.

Respectfully submitted,

Date: August 5, 1998



Daniel Hansburg
Kimeragen, Inc.
300 Pheasant Run
Newtown, PA 18940
215-504-4444 X-160

THE USE OF MIXED DUPLEX OLIGONUCLEOTIDES TO EFFECT LOCALIZED GENETIC CHANGES IN PLANTS

1. FIELD OF THE INVENTION

The field of the present invention relates to methods for the improvement of existing lines of plants and to the development of new lines having desired traits. The previously available methods of obtaining genetically altered plants by recombinant DNA technology enabled the introduction of preconstructed exogenous genes in random, atopic positions, so-called transgenes. In contrast the present invention allows the skilled practitioner to make a specific alteration of a specific pre-existing gene of a plant. The invention utilizes duplex oligonucleotides having a mixture of RNA-like nucleotides and DNA-like nucleotides to effect the alterations, hereafter "mixed duplex oligonucleotides" or MDON.

2. BACKGROUND TO THE INVENTION

2.1 MDON and Their Use to Effect Specific Genetic Alterations

Mixed duplex oligonucleotides (MDON) and their use to effect genetic changes in eukaryotic cells are described in United States patent No. 5,565,350 to Kmiec (Kmiec I). Kmiec I discloses *inter alia* MDON having two strands, in which a first strand contains two segments of at least 8 RNA-like nucleotides that are separated by a third segment of from 4 to about 50 DNA-like nucleotides, termed an "interposed DNA segment." The nucleotides of the first strand are base paired to DNA-like nucleotides of a second strand. The first and second strands are additionally linked by a segment of single stranded nucleotides so that the first and second strands are parts of a single oligonucleotide chain. Kmiec I further teaches a method for introducing specific genetic alterations into a target gene. According to Kmiec I, the sequences of the RNA segments are selected to be homologous, i.e., identical, to the sequence of a first and a second fragment of the target gene. The sequence of the interposed DNA segment is homologous with the sequence of the target gene between the first and second fragment except for a region of difference, termed the "heterologous region." The heterologous region can effect an insertion or deletion, or can contain one or

more bases that are mismatched with the sequence of target gene so as to effect a substitution. According to Kmiec I, the sequence of the target gene is altered as directed by the heterologous region, such that the target gene becomes homologous with the sequence of the MDON. Kmiec I specifically teaches that ribose and 2'-O-methylribose, i.e., 2'-methoxyribose, containing nucleotides can be used in MDON and that naturally-occurring deoxyribose-containing nucleotides can be used as DNA-like nucleotides.

United States patent application Serial No. 08\664,487, filed June 17, 1996, now U.S. patent No. 5,731,181 (Kmiec II) does specifically disclose the use of MDON to effect genetic changes in plant cells and discloses further examples of analogs and derivatives of RNA-like and DNA-like nucleotides that can be used to effect genetic changes in specific target genes.

Scientific publications disclosing uses of MDON having interposed DNA segments include Yoon, et al., 1996, *Proc. Natl. Acad. Sci.* 93:2071-2076 and Cole-Straus, A. et al., 1996, *SCIENCE* 273 :1386-1389. The scientific publications disclose that rates of mutation as high as about one cell in ten can be obtained using liposomal mediated delivery. However, the scientific publications do not disclose that MDON can be used to make genetic changes in plant cells.

The present specification uses the term MDON, which should be understood to be synonymous with the terms "chimeric mutation vector," "chimeric repair vector" and "chimeraplast" which are used elsewhere.

2.2 Transgenic Plant Cells and the Generation of Plants from Transgenic Plant Cells

Of the techniques taught by Kmiec I and II for delivery of MDON into the target cell, the technique that is most applicable for use with plant cells is the electroporation of protoplasts. The regeneration of fertile plants from protoplast cultures has been reported for certain species of dicotyledonous plants, e.g., *Nicotiana tabacum* (tobacco), United States Patent 5,231,019 and Fromm, M.E., et al., 1988, *Nature* **312**, 791, and soybean variety *Glycine max*, WO 92/17598 to Widholm, J.M. However, despite the reports of isolated successes using non-transformed cells, Prioli, L.M., et al., *Bio/Technology* 7, 589, Shillito, R.D., et al., 1989, *Bio/Technology* 7, 581, the regeneration of fertile monocotyledonous plants from transformed protoplast

cultures is not regarded as obtainable with application of routine skill. Frequently, transformed protoplasts of monocotyledonous plants result in non-regenerable tissue or, if the tissue is regenerated the resultant plant is not fertile.

Other techniques to obtain transformed plant cells by introducing kilobase-sized plasmid DNA into plant cells having intact or partially intact cell walls have been developed. United States patent No. 4,945,050, No. 5,100,792 and No. 5,204,253 concern the delivery of plasmids into intact plant cells by adhering the plasmid to a microparticle that is ballistically propelled across the cell wall, hereafter "biolistically transformed" cell. For example U.S. patent No. 5,489,520 describes the regeneration of a fertile maize plant from a biolistically transformed cell. Other techniques for the introduction of plasmid DNA into suspensions of plant cells having intact cell walls include the use of silicon carbide fibers to pierce the cell wall, see U.S. patent No. 5,302,523 to Coffee R., and Dunwell, J.M.

A technique that allows for the electroporation of maize cells having a complex cell wall is reported in U.S. patent No. 5,384,253 to Krzyzek, Laursen and P.C. Anderson. The technique uses a combination of the enzymes endopectin lyase (E.C. 3.2.1.15) and endopolygalacturonase (E.C. 4.2.2.3) to generate transformation competent cells that can be more readily regenerated into fertile plants than true protoplasts. However, the technique is reported to be useful only for F1 cell lines from the cross of line A188 x line B73.

3. SUMMARY OF THE INVENTION

The present invention provides new methods of use of the MDON that are particularly suitable for use in such plant cells.

Thus one aspect of the invention is techniques to adhere MDON to particles which can be projected through the cell wall to release the MDON within the cell in order to cause a mutation in a target gene of the plant cell. The mutations that can be introduced by this technique are mutations that confer a growth advantage to the mutated cells under appropriate conditions and mutations that cause a phenotype that can be detected by visual inspection. Such mutations are termed "selectable mutations."

In a further embodiment the invention encompasses a method of introducing a

mutation other than a selectable mutation into a target gene of a plant cell by a process which includes the steps of introducing a mixture of a first MDON that introduces a selectable mutation in the plant cell and a second MDON that causes the non-selectable mutation.

The invention further encompasses the culture of the cells mutated according to the foregoing embodiments of the invention so as to obtain a plant that produces seeds, henceforth a "fertile plant," and the production of seeds and additional plants from such a fertile plant.

The invention further encompasses fertile plants having novel characteristics which can be produced by the methods of the invention.

4. DETAILED DESCRIPTION OF THE INVENTION

4.1 Recombinagenic Oligonucleobases and Mixed Duplex OligoNucleotides

The invention can be practiced with MDON having the conformations and chemistries described in Kmiec I or in Kmiec II, which are hereby incorporated by reference. The MDON of Kmiec I and/or Kmiec II contain two complementary strands, one of which contains at least one segment of RNA-type nucleotides (an "RNA segment") that are base paired to DNA-type nucleotides of the other strand.

Kmiec II discloses that purine and pyrimidine base-containing non-nucleotides can be substituted for nucleotides. Commonly assigned U.S. patent applications Serial No. 09/078,063, filed May 12, 1998, and Serial No. 09/078,064, filed May 12, 1998, which are each hereby incorporated in their entirety, disclose additional molecules that can be used for the present invention. The term "recombinagenic oligonucleobase" is used herein to denote the molecules that can be used in the present invention. Recombinagenic oligonucleobases include MDON, non-nucleotide containing molecules taught in Kmiec II and the molecules taught in the above noted commonly assigned patent applications.

In a preferred embodiment the RNA-type nucleotides of the MDON are made Rnase resistant by having replacing the 2'-hydroxyl with a fluoro, chloro or bromo functionality or by placing a substituent on the 2'-O. Suitable substituents include the

substituents taught by the Kmiec II, C₁₋₆ alkane. Alternative substituents include the substituents taught by U.S. Patent No. 5,334,711 (Sproat) and the substituents taught by patent publications EP 629 387 and EP 679 657 (collectively, the Martin Applications), which are hereby incorporated by reference. As used herein a 2' - fluoro, chloro or bromo derivative of a ribonucleotide or a ribonucleotide having a 2' - OH substituted with a substituent described in the Martin Applications or Sproat is termed a "2'-Substituted Ribonucleotide." As used herein the term "RNA-type nucleotide" means a 2'-hydroxyl or 2'-Substituted Nucleotide that is linked to other nucleotides of a MDON by an unsubstituted phosphodiester linkage or any of the non-natural linkages taught by Kmiec I or Kmiec II. As used herein the term "deoxyribo-type nucleotide" means a nucleotide having a 2'-H, which can be linked to other nucleotides of a MDON by an unsubstituted phosphodiester linkage or any of the non-natural linkages taught by Kmiec I or Kmiec II.

A particular embodiment of the invention comprises MDON that are linked solely by unsubstituted phosphodiester bonds. Alternatively embodiments comprise linkage by substituted phosphodiesters, phosphodiester derivatives and non-phosphorus-based linkages as taught by Kmiec II. A further particular embodiment comprises MDON wherein each RNA-type nucleotide is a 2'-Substituted Nucleotide. Particular preferred embodiments of 2'-Substituted Ribonucleotides are 2'-fluoro, 2'-methoxy, 2'-propyloxy, 2'-allyloxy, 2'-hydroxyethyloxy, 2'-methoxyethyloxy, 2'-fluoropropyloxy and 2'-trifluoropropyloxy substituted ribonucleotides. In more preferred embodiments of 2'-Substituted Ribonucleotides are 2'-fluoro, 2'-methoxy, 2'-methoxyethyloxy, and 2'-allyloxy substituted nucleotides. In one embodiment the MDON oligomer is linked by unsubstituted phosphodiester bonds.

Although MDON having only a single type of 2'-substituted RNA-type nucleotide are more conveniently synthesized, the invention can be practiced with MDON having two or more types of RNA-type nucleotides. The function of an RNA segment may not be affected by an interruption caused by the introduction of a deoxynucleotide between two RNA-type trinucleotides, accordingly, the term RNA segment encompasses such an "interrupted RNA segment." An uninterrupted RNA segment is termed a contiguous RNA segment. In an alternative embodiment an RNA segment can contain alternating RNase-resistant and unsubstituted 2'-OH nucleotides.

09:55:30 05/26/2016

The MDON of the invention preferably have fewer than 100 nucleotides and more preferably fewer than 85 nucleotides, but more than 50 nucleotides. The first and second strands are Watson-Crick base paired. In one embodiment the strands of the MDON are covalently bonded by a linker, such as a single stranded hexa, penta or tetranucleotide so that the first and second strands are segments of a single oligonucleotide chain having a single 3' and a single 5' end. The 3' and 5' ends can be protected by the addition of a "hairpin cap" whereby the 3' and 5' terminal nucleotides are Watson-Crick paired to adjacent nucleotides. A second hairpin cap can, additionally, be placed at the junction between the first and second strands distant from the 3' and 5' ends, so that the Watson-Crick pairing between the first and second strands is stabilized.

The first and second strands contain two regions that are homologous with two fragments of the target gene, i.e., have the same sequence as the target gene. A homologous region contains the nucleotides of an RNA segment and may contain one or more DNA-type nucleotides of connecting DNA segment and may also contain DNA-type nucleotides that are not within the intervening DNA segment. The two regions of homology are separated by, and each is adjacent to, a region having a sequence that differs from the sequence of the target gene, termed a "heterologous region." The heterologous region can contain one, two or three mismatched nucleotides. The mismatched nucleotides can be contiguous or alternatively can be separated by one or two nucleotides that are homologous with the target gene. Alternatively, the heterologous region can also contain an insertion or one, two, three or of five or fewer nucleotides. Alternatively, the sequence of the MDON may differ from the sequence of the target gene only by the deletion of one, two, three, or five or fewer nucleotides from the MDON. The length and position of the heterologous region is, in this case, deemed to be the length of the deletion, even though no nucleotides of the MDON are within the heterologous region. The distance between the fragments of the target gene that are complementary to the two homologous regions is identically the length of the heterologous region when a substitution or substitutions is intended. When the heterologous region contains an insertion, the homologous regions are thereby separated in the MDON farther than their complementary homologous fragments are in the gene, and the converse is applicable

when the heterologous region encodes a deletion.

The RNA segments of the MDON are each a part of a homologous region, i.e., a region that is identical in sequence to a fragment of the target gene, which segments together preferably contain at least 13 RNA-type nucleotides and preferably from 16 to 25 RNA-type nucleotides or yet more preferably 18-22 RNA-type nucleotides or most preferably 20 nucleotides. In one embodiment, RNA segments of the homology regions are separated by and adjacent to, i.e., "connected by" an intervening DNA segment. In one embodiment, each nucleotide of the heterologous region is a nucleotide of the intervening DNA segment. An intervening DNA segment that contains the heterologous region of a MDON is termed a "mutator segment."

Commonly assigned U.S. patent application Serial No. 09/078,063, filed May 12, 1998, and Serial No. 09/078,064, filed May 12, 1998, disclose a type of duplex recombinagenic oligonucleobase in which a strand has a sequence that is identical to that of the target gene and only the sequence of the "complementary" strand contains a heterologous region. This configuration results in one or more mismatched bases or a "heteroduplex" structure. The heterologous region of the heteroduplex recombinagenic oligonucleobases that are useful in the present invention is located in the strand that contains the deoxynucleotides. In one embodiment, the heterologous region is located on the strand that contains the 5' terminal nucleotide.

4.2 The Location and Type of Mutation Introduced by a MDON

Frequently, the design of the MDON for use in plant cells must be modified from the designs taught in Kmiec I and II. In mammalian and yeast cells, the genetic alteration introduced by a MDON that differs from the target gene at one position is the replacement of the nucleotide in the target gene at the mismatched position by a nucleotide complementary to the nucleotide of the MDON at the mismatched position. By contrast, in plant cells there can be an alteration of the nucleotide one base 5' to the mismatched position on the strand that is complementary to the strand that contains the DNA mutator segment. The nucleotide of the target gene is replaced by a nucleotide complementary to the nucleotide of the DNA mutator segment at the mismatched position. Consequently, the mutated target gene differs from the MDON at two positions.

The mutations introduced into the target gene by a MDON are located between the regions of the target gene that are homologous with the ribonucleotide portion of the homology regions of the MDON, henceforth the "RNA segments." The specific mutation that is introduced depends upon the sequence of the heterologous region. An insertion or deletion in the target gene can be introduced by using a heterologous region that contains an insertion or deletion, respectively. A substitution in the target gene can be obtained by using a MDON having a mismatch in the heterologous region of the MDON. In the most frequent embodiments, the mismatch will convert the existing base of the target gene into the base that is complementary to the mismatched base of the MDON. The location of the substitution in the target gene can be either at the position that corresponds to the mismatch or, more frequently, the substitution will be located at the position on the target strand immediately 5' to the position of the mismatch, i.e., complementary to the position of the MDON immediately 3' of the mismatched base of the MDON.

The relative frequency of each location of the mismatch-caused substitution will be characteristic of a given gene and cell type. Thus, those skilled in the art will appreciate that a preliminary study to determine the location of substitutions in the gene of particular interest is generally indicated, when the location of the substitution is critical to the practice of the invention.

4.3 The Delivery of MDON by Microcarriers and Microfibers

The use of metallic microcarriers (microspheres) for introducing large fragments of DNA into plant cells having cellulose cell walls by projectile penetration is well known to those skilled in the relevant art (henceforth biolistic delivery). United States patents No. 4,945,050, No. 5,100,792 and No. 5,204,253 concern general techniques for selecting microcarriers and devices for projecting them.

The conditions that are used to adhere DNA fragments to the microcarriers are not suitable for the use of MDON. The invention provides techniques for adhering sufficient amounts of MDON to the microcarrier so that biolistic delivery can be employed. In a suitable technique, ice cold microcarriers (60 mg/ml), MDON (60 mg/ml) 2.5 M CaCl_2 and 0.1 M spermidine are added in that order; the mixture gently agitated, e.g., by vortexing, for 10 min and allowed to stand at room temperature for

10 min, whereupon the microcarriers are diluted in 5 volumes of ethanol, centrifuged and resuspended in 100% ethanol. Good results can be obtained with a concentration in the adhering solution of 8-10 $\mu\text{g}/\mu\text{l}$ microcarriers, 14-17 $\mu\text{g}/\text{ml}$ MDON, 1.1-1.4 M CaCl_2 and 18-22 mM spermidine. Optimal results were observed under the conditions of 8 $\mu\text{g}/\mu\text{l}$ microcarriers, 16.5 $\mu\text{g}/\text{ml}$ MDON, 1.3 M CaCl_2 and 21 mM spermidine.

MDON can also be introduced into plant cells for the practice of the invention using microfibers to penetrate the cell wall and cell membrane. U.S. Patent No. 5,302,523 to Coffee et al. describes the use of $30 \times 0.5 \mu\text{m}$ and $10 \times 0.3 \mu\text{m}$ silicon carbide fibers to facilitate transformation of suspension maize cultures of Black Mexican Sweet. Any mechanical technique that can be used to introduce DNA for transformation of a plant cell using microfibers can be used to deliver MDON for transmutation.

A suitable technique for microfiber delivery of MDON is as follows. Sterile microfibers (2 μg) are suspended in 150 μl of plant culture medium containing about 10 μg of MDON. A suspension culture is allowed to settle and equal volumes of packed cells and the sterile fiber/MDON suspension are vortexed for 10 minutes and plated. Selective media are applied immediately or with a delay of up to about 120 hours as is appropriate for the particular trait.

The techniques that can be used to deliver MDON to transmute nuclear genes can also be used to cause transmutation of the genes of a plastid of a plant cell. Plastid transformation of higher plants by biolistic delivery of a plasmid followed by an illegitimate recombinatorial insertion of the plasmid is well known to those skilled in the art. Svab, Z., et al., 1990, Proc. Natl. Acad. Sci. **87**, 8526-8530. The initial experiments showed rates of transformation that were between 10-fold and 100-fold less than the rate of nuclear transformation. Subsequent experiments showed that rates of plasmid transformation comparable to the rate of nuclear transformation could be achieved by use of a dominant selectable trait such as a bacterial aminoglycoside 3'-adenosyltransferase gene, which confers spectinomycin resistance. Svab, Z., & Maliga, P., 1993, Proc. Natl. Acad. Sci. **90**, 913-917.

According to the invention MDON for the transmutation of plastid genes can be introduced into plastids by the same techniques as above. When the mutation

desired to be introduced is a selectable mutation the MDON can be used alone. When the desired mutation is non-selectable the relevant MDON can be introduced along with a MDON that introduces a selectable plastid mutation, e.g., a mutation in the psbA gene that confers triazine resistance, or in combination with a linear or circular plasmid that confers a selectable trait.

The foregoing techniques can be adapted for use with recombinagenic oligonucleobases other than MDON.

4.4 Protoplast Electroporation

In an alternative embodiment the recombinagenic oligonucleobase can be delivered to the plant cell by electroporation of a protoplast derived from a plant part. The protoplasts are formed by enzymatic treatment of a plant part, particularly a leaf, according to techniques well known to those skilled in the art. See, e.g., Gallois et al., 1996, in *Methods in Molecular Biology* 55, 89-107 (Humana Press, Totowa, NJ). The protoplasts need not be cultured in growth media prior to electroporation.

Suitable conditions for electroporation are 3×10^5 protoplasts in a total volume of 0.3 ml with a concentration of MDON of between 0.6 - 4 $\mu\text{g/mL}$.

4.5 The Introduction of Mutations

The invention can be used to effect genetic changes, herein "transmutate," in plant cells. In an embodiment the plant cells have cell walls, i.e., are other than protoplasts.

The use of MDON to transmutate plant cells can be facilitated by co-introducing a trait that allows for the ready differentiation and separation of cells (hereafter "selection") into which MDON have been introduced from those that have not. In one embodiment of the invention the selection is performed by forming a mixture of MDON and a plasmid that causes the transient expression of a gene that confers a selectable trait, i.e., one that permits survival under certain conditions, e.g., a kanamycin resistance gene. Under these circumstances elimination of cells lacking the selectable trait removes the cells into which MDON were not introduced. The use of a transient expression plasmid to introduce the selectable trait allows for the successive introduction of multiple genetic changes into a plant cell by repeatedly

using a single standardized selection protocol.

In an alternative embodiment transmutation can be used to introduce a selectable trait. A mixture of a first MDON that causes a selectable mutation in a first target gene and a second MDON that causes a non-selectable mutation in a second target gene is prepared. According to the invention, at least about 1% of the cells having the selectable mutation will be found to also contain a mutation in the second target gene that was introduced by the second MDON. More frequently at least about 10% of the cells having the selectable mutation will be found to also contain a mutation in the second target gene.

One use of this embodiment of the invention is the investigation of the function of a gene-of-interest. A mixture is provided of a MDON that causes a selectable mutation and a MDON that causes a mutation that would be expected to "knock-out" the gene-of-interest, e.g., the insertion of a stop codon or a frameshift mutation. Cells in which one or more copies of the gene-of-interest have been knocked out can be recovered from the population having the selectable mutation. Such cells can be regenerated into a plant so that the function of the gene-of-interest can be determined.

A selectable trait can be caused by any mutation that causes a phenotypic change that can produce a selective growth advantage under the appropriate selective conditions or a phenotypic change that can be readily observed, such as change in color of the plant cells growing in a callus. The selectable trait can itself be a desirable traits, e.g., herbicide resistance, or the selectable trait can be used merely to facilitate the isolation of plants having a non-selectable trait that was introduced by transmutation. A desired nonselectable trait can be introduced into a cell by using a mixture of the MDON that causes the desired mutation and the MDON that causes the selectable mutation, followed by culture under the selecting conditions. Selection according to this scheme has the advantage of ensuring that each selected cell not only received the mixture of MDONs, but also that the cell which received the mixture was then susceptible to transmutation by a MDON.

A mutation that causes a lethal phenotypic change under the appropriate conditions, termed a negatively selectable mutation, can also be used in the present invention. Such mutations cause negatively selectable traits. Negatively selectable

traits can be selected by making replica plates of the transmutated cells, selecting one of the replicas and recovering the transmutated cell having the desired property from the non-selected replica.

4.6 Specific Genes That Can Be Transmutated to Create Selectable Traits

In one embodiment of the invention a MDON is used to introduce a mutation into an Acetolactate synthase (ALS) gene, which is also termed the aceto-hydroxy amino acid synthase (AHAS) gene. Sulfonylurea herbicides and imidazoline herbicides are inhibitors of the wild type ALS enzymes. Dominant mutations that render plants resistant to the actions of sulfonylureas and imidazolines have been described. See U.S. Patent Nos. 5,013,659 and 5,378,824 (Bedbrook) and Rajasekaran K., et al., 1996, Mol. Breeding **2**, 307-319 (Rajasekaran). Bedbrook at Table 2 describes several mutations (hereafter, a "Bedbrook Mutation") that were found to render yeast ALS enzymes resistant to sulfonylurea herbicides. Bedbrook states that each of the Bedbrook mutations makes a plant resistant to sulfonylurea and imidazoline herbicides when introduced into a plant ALS gene. It is understood that in most plants the gene encoding ALS has been duplicated. A mutation can be introduced into any allele of either ALS gene.

Three of the Bedbrook mutations were, in fact, shown to confer herbicide resistance in a plant, namely the substitutions Pro→Ala¹⁹⁷, Ala→Asp²⁰⁵ and Trp→Leu⁵⁹¹. Rajasekaran reports that mutations Trp→Ser⁵⁹¹ caused resistance to sulfonylurea and imidazoline and that Ser→Asn⁶⁶⁰ caused resistance to imidazoline herbicides. The results of Rajasekaran are reported herein using the sequence numbering of Bedbrook. Those skilled in the art will understand that the ALS genes of different plants are of unequal lengths. For clarity, a numbering system is used in which homologous positions are designated by the same position number in each species. Thus, the designated position of a mutation is determined by the sequence that surrounds it. For example, the mutation Trp→Ser⁵⁹¹ of Rajasekaran is at residue 563 of the cotton ALS gene but is designated as position 591 of Bedbrook because the mutated Trp is surrounded by the sequence that surrounds Trp⁵⁹¹ in Table 2 of Bedbrook. According to the invention any substitution for the naturally occurring amino acid at position 660 or one of the positions listed in Table 2 of Bedbrook, which is hereby incorporated by

reference, can be used to make a selectable mutation in the ALS gene of a plant.

In a further embodiment of the invention the selectable mutation can be a mutation in the chloroplast gene psbA that encodes the D1 subunit of photosystem II, see Hirschberg, J., et al., 1984, Z. Naturforsch. **39**, 412-420 and Ohad, N., & Hirschberg, J., The Plant Cell **4**, 273-282. Hirschberg et al. reports that the mutation Ser→Gly²⁶⁴ results in resistance to triazine herbicides, e.g., 2-Cl-4-ethylamino-6-isopropylamino-s-triazine (Atrazine). Other mutations in the psbA gene that cause Atrazine herbicide resistance are described in Erickson J.M., et al., 1989, Plant Cell **1**, 361-371, (hereafter an "Erickson mutation"), which is hereby incorporated by reference. The use of the selectable trait caused by an Erickson mutation is preferred when it is desired to introduce a second new trait into a chloroplast.

The scientific literature contains further reports of other mutations that produce selectable traits. Ghislain M., et al., 1995, The Plant Journal **8**, 733-743, describes a Asn→Ile¹⁰⁴ mutation in the *Nicotiana sylvestris* dihydrodipicolinate synthase (DHDPS, EC 4.2.1.52) gene that results in resistance to S-(2-aminoethyl)L-cysteine. Mourad, G., & King, J., 1995, Plant Physiology **109**, 43-52 describes a mutation in the threonine dehydratase of *Arabidopsis thaliana* that results in resistance to L-O-methylthreonine. Nelson, J.A.E., et al., 1994, Mol. Cell. Biol. **14**, 4011-4019 describes the substitution of the C-terminal Leu of the S14/rp59 ribosomal protein by Pro, which causes resistance to the translational inhibitors cryptoluerine and emetine. In further embodiments of the invention, each of the foregoing mutations can be used to create a selectable trait. Each of Ghislain, Mourad and Nelson are hereby incorporated by reference.

4.7 Genes That Can Be Mutated to Create Desirable Non-selectable Traits

Example 1

MALE STERILITY

Certain commercially grown plants are routinely grown from hybrid seed including corn (maize, *Zea mays*), tomatoes and most other vegetables. The production of hybrid seed requires that plants of one purebred line be pollinated only by pollen from another purebred line, i.e., that there be no self pollination. The removal of the pollen-producing organs from the purebred parental plants is a

laborious and expensive process. Therefore, a mutation that induces male-sterility i.e., suppresses pollen production or function, would obviate the need for such process.

Several genes have been identified that are necessary for the maturation or function of pollen but are not essential for other processes of the plant. Chalcone synthase (*chs*) is the key enzyme in the synthesis of flavonoids, which are pigments found in flowers and pollen. Inhibition of *chs* by the introduction of a *chs* antisense expressing gene in the petunia results in male sterility of the plant. Van der Meer, I.M., et al., 1992, *The Plant Cell* **4**, 253-262. There is a family of *chs* genes in most plants. See, e.g., Koes, R.E., et al., 1989, *Plant Mol. Biol.* **12**, 213-226. Likewise disruption of the chalcone synthase gene in maize by insertion of a transposable element results in male sterility. Coe, E.H., *J. Hered.* **72**, 318-320. The structure of maize chalcone synthase and a duplicate gene, *whp*, is given in Franken, P., et al., 1991, *EMBO J.* **10**, 2605-2612. Typically in plants each member of a multigene family is expressed only in a limited range of tissues. Accordingly, the present embodiment of the invention requires that in species having multiple copies of chalcone synthase genes, the particular *chs* gene or genes expressed in the anthers be identified and interrupted by introduction of a frameshift, and one or more in-frame termination codons or by interruption of the promoter.

A second gene that has been identified as essential for the production of pollen is termed *Lat52* in tomato. Muschietti, J., et al., 1994, *The Plant Journal* **6**, 321-338. *LAT52* is a secreted glycoprotein that is related to a trypsin inhibitor. Homologs of *Lat52* have been identified in maize (termed *Zm13*, Hanson D.D., et al., 1989 *Plant Cell* **1**, 173-179; Twell D., et al., 1989, *Mol. Gen. Genet.* **217**, 240-245), rice (termed *Ps1*, Zou J., et al., 1994 *Am. J. Bot.* **81**, 552-561 and olive (termed *Ole e 1*, Villalba, M., et al., 1993, *Eur. J. Biochem.* **276**, 863-869). Accordingly, the present embodiment of the invention provides for a method of obtaining male sterility by the interruption of the *Lat52/Zm13* gene or its homologs by the introduction of a frameshift, one or more in-frame termination codons or by interruption of the promoter.

A third gene that has been identified as essential for the production of pollen is the gene that encodes phenylalanine ammonium lyase (PAL, EC 4.3.1.5). PAL is an essential enzyme in the production of both phenylpropanoids and flavonoids.

Because phenylpropanoids are a precursor to lignins, which can be an essential for the resistance to disease in the preferred embodiment a PAL isozyme that is expressed only in the anther is identified and interrupted to obtain male sterility.

Example 2 ALTERATION OF CARBOHYDRATE METABOLISM OF TUBERS

Once harvested, potato tubers are subject to disease, shrinkage and sprouting during storage. To avoid these losses the storage temperature is reduced to 35-40° F. However, at reduced temperatures, the starch in the tubers undergoes conversion to sugar, termed "cold sweetening", which reduces the commercial and nutritional value of the tuber. Two enzymes are critical for the cold sweetening process: acid invertase and UDP-glucose pyrophosphorylase. Zrenner, R., et al., 1996, *Planta* **198**, 246-252 and Spychalla, J.P., et al., 1994, *J. Plant Physiol.* **144**, 444-453, respectively. The sequence of potato acid invertase is found in EMBL database Accession No. X70368 (SEQ ID NO. 1) and the sequence of the potato UDP Glucose pyrophosphorylase is reported by Katsube, T. et al., 1991, *Biochem.* **30**, 8546-8551. Accordingly, the present embodiment of the invention provides for a method of preventing cold sweetening by the interruption of the acid invertase or the UDP glucose phosphorylase gene by introduction of a frameshift, one or more in-frame termination codons or by interruption of the promoter.

Example 3 REDUCTION IN POST HARVEST BROWNING DUE TO PPO

Polyphenol oxidase (PPO) is the major cause of enzymatic browning in higher plants. PPO catalyzes the conversion of monophenols to o-diphenols and of o-dihydroxyphenols to o-quinones. The quinone products then polymerize and react with amino acid groups in the cellular proteins, which results in discoloration. The problem of PPO induced browning is routinely addressed by the addition of sulfites to the foods, which has been found to be associated with some possible health risk and consumer aversion. PPO normally functions in the defense of the plant to pathogens or insect pests and, hence, is not essential to the viability of the plant. Accordingly, the present embodiment of the invention provides for a method of preventing enzymatic browning by the interruption of the PPO gene by introduction of a frameshift, one or more in-frame termination codons or by interruption of the promoter

in apple, grape, avocado, pear and banana.

The number of PPO genes in the genome of a plant is variable; in tomatoes and potatoes PPO forms a multigene family. Newman, S.M., et al., 1993, *Plant Mol. Biol.* **21**, 1035-1051, Hunt M.D., et al., 1993, *Plant Mol. Biol.* **21**, 59-68; Thygesen, P.W., et al., 1995, *Plant Physiol.* **109**, 525-531. The grape contains only a single PPO gene. Dry, I.B., et al., 1994, *Plant Mol. Biol.*, **26**, 495-502. When the plant species of interest contains multiple copies of PPO genes it is essential that the PPO gene that is normally expressed in the commercial product be interrupted. For example, only one PPO gene is expressed in potatoes of harvestable size, which gene is termed POT32 and its sequence is deposited in GENBANK accession No. U22921 (SEQ ID NO. 2), which sequence is incorporated by reference. The other potato PPO isozymes have been sequenced and the sequences deposited so that one skilled in the art can design a MDON that specifically inactivates POT32.

Example 4 REDUCTION OF LIGNIN IN FORAGE CROPS AND WOOD PULP

Lignin is a complex heterogeneous aromatic polymer, which waterproofs higher plants and strengthens their cell walls. Lignin arises from the random polymerization of free radicals of phenylpropanoid monolignins. Lignins pose a serious problem for the paper industry because their removal from wood pulp involves both monetary and environmental costs. Similarly, the lignin content of forage crops limits their digestibility by ruminants. Indeed, naturally occurring mutations, termed "brown mid-rib" in sorghum, Porter, KS, et al., 1978, *Crop Science* **18**, 205-218, and maize, Lechtenberg, V.L., et al., 1972, *Agron. J.* **64**, 657-660, have been identified as having reduced lignin content and tested as feed for cattle.

The brown mid-rib mutation in maize involves the O-methyl transferase gene. Vignol, F., et al., 1995, *Plant Cell* **7**, 407-416. The O-methyltransferase genes of a number of plant species have been cloned: Burgos, R.C., et al., 1991, *Plant Mol. Biol.* **17**, 1203-1215 (aspen); Gowri, G., et al., 1991, *Plant Physiol.* **97**, 7-14 (alfalfa, *Medicago sativa*) and Jaeck, E., et al., 1992, *Mol. Plant-Microbe Interact.* **4**, 294-300 (tobacco) (SEQ ID No. 3 and SEQ ID No. 4). Thus, one aspect of the present embodiment is the interruption of the O-methyltransferase gene to reproduce a brown mid-rib phenotype in any cultivar of maize or sorghum and in other species of forage

crops and in plants intended for the manufacture of wood pulp.

A second gene that is involved in lignin production is the cinnamyl alcohol dehydrogenase (CAD) gene, which has been cloned in tobacco. Knight, M.E., 1992, Plant Mol. Biol. **19**, 793-801 (SEQ ID No. 5 and SEQ ID No. 6). Transgenic tobacco plants making a CAD antisense transcript have reduced levels of CAD and also make a lignin that is more readily extractable, apparently due to an increase in the ratio of syringyl to guaiacyl monomers and to the increased incorporation of aldehyde monomers relative to alcohol residues. Halpin, C., et al., 1994, The Plant Journal **6**, 339-350. Accordingly, an embodiment of the invention is the interruption of the CAD gene of forage crops such as alfalfa, maize, sorghum and soybean and of paper pulp trees such as short-leaf pine (*Pinus echinata*) long-leaf pine (*Pinus palustris*) slash pine (*Pinus elliotii*), loblolly pine (*Pinus taeda*), yellow-poplar (*Liriodendron tulipifera*) and cotton wood (*Populus sp.*) by introduction of a frameshift, one or more in-frame termination codons or by interruption of the promoter.

Example 5 THE REDUCTION IN UNSATURATED AND POLYUNSATURATED LIPIDS IN OIL SEEDS

The presence of unsaturated fatty acids, e.g., oleic acid, and polyunsaturated fatty acids, e.g., linoleic and linolenic acids, in vegetable oil from oil seeds such as rape, peanut, sunflower and soybean causes the oils to oxidize, on prolonged storage and at high temperatures. Consequently, vegetable oil is frequently hydrogenated. However, chemical hydrogenation causes transhydrogenation, which produces non-naturally occurring stereo-isomers, which are believed to be a health risk.

Fatty acid synthesis proceeds by the synthesis of the saturated fatty acid on an acyl carrier protein (ACP) followed by the action of desaturases that remove the hydrogen pairs. Consequently, it would be desirable to inhibit the activity of these desaturase enzymes in oil seeds.

The first enzyme in the synthesis of oleic acid is stearoyl-ACP desaturase (EC 1.14.99.6). The stearoyl-ACP desaturases from safflower and castor bean have been cloned and sequenced. Thompson, G.A., et al., 1991, Proc. Natl. Acad. Sci. **88**, 2578-2582 (SEQ ID No. 7); Shanklin, J., & Somerville, C., 1991, Proc. Natl. Acad. Sci. **88**, 2510-2514 (SEQ ID No. 8); Knutzon, D.S., et al., 1991, Plant Physiology **96**, 344-

345. Accordingly, one embodiment of the present invention is the interruption of the stearyl-ACP desaturase gene of oil seed crops such as soybean, safflower, sunflower, soy, maize and rape by introduction of a frameshift, one or more in-frame termination codons or by interruption of the promoter.

A second enzyme that can be interrupted according to the present invention is ω -3 fatty acid desaturase (ω -3 FAD) the enzyme that converts linoleic acid, a diene, to linolenic acid, a triene. There are two ω -3 FAD isozymes in *Arabidopsis thaliana* and, those skilled in the art expect, in most other plants. One isozyme is specific for plastids and is the relevant isozyme for the synthesis of the storage oils of seeds. The other is microsome specific. The cloning of the *Arabidopsis thaliana* plastid ω -3 FAD is reported by Iba., K. et al., 1993, J. Biol. Chem. **268**, 24099-24105 (SEQ ID No. 9). Accordingly an embodiment of the invention is the interruption of the plastid ω -3 FAD gene of oil seed crops such as soybean, safflower, sunflower, soy, maize and rape by introduction of a frameshift, an in-frame termination codon or by interruption of the promoter.

Example 6

INACTIVATION OF S ALLELES TO PERMIT INBRED LINES

Certain plant species have developed a mechanism to prevent self-fertilization. In these species, e.g., wheat and rice, there is a locus, termed S, which has multiple alleles. A plant that expresses an S allele cannot be fertilized by pollen expressing the same S allele. Lee, H-K., et al., 1994, Nature **367**, 560-563; Murfett, J., et al., 1994, Nature **367**, 563. The product of the S locus is an RNase. McClure, B.A., et al., 1989, Nature **342**, 955-957. The product of the S locus is not essential for the plant. Accordingly, an embodiment of the invention is the interruption of genes of the S locus to permit the inbreeding of the plant by introduction of a frameshift, one or more in-frame termination codons or by interruption of the promoter.

Example 7

ETHYLENE INSENSITIVITY

Ethylene is a gaseous plant hormone that is involved in plant growth and development. An unwanted aspect of ethylene's action is the over-ripening of fruit, vegetables and the wilting of flowers that results in rotting and loss. The ethylene

receptor of *Arabidopsis thaliana* has been cloned and is termed ETR-1. Chang, C., et al., 1993, Science **262**, 539-544 (SEQ ID No. 10). A mutant, Cys→Tyr⁶⁵, results in a dominant insensitivity to ethylene. Transgenic tomato plants expressing the *Arabidopsis thaliana* mutant ETR-1 also showed an insensitivity to ethylene, indicating that the Cys→Tyr⁶⁵ mutation would be a dominant suppressor of ethylene action in most plant species. Accordingly one aspect of the present embodiment of the invention is the insertion of the Cys→Tyr⁶⁵ mutation into the ETR-1 gene so as to extend the life span of the mutated fruit vegetable or flower.

In a further aspect of the present embodiment, the preservation of the fruit or flower can be achieved by interrupting one of the genes that encode the enzymes for ethylene synthesis: namely 1-aminocyclopropane-1-carboxylic acid synthase (ACC synthase) and ACC oxidase. For this embodiment of the invention the amount of ethylene synthesis can be eliminated entirely, so that ripening is produced by exogenous ethylene or some amount of ethylene production can be retained so that the fruit ripens spontaneously, but a has a prolonged storage life. Accordingly, it is anticipated that the interruption of one allele of either the ACC synthase or the ACC oxidase gene can result in an useful reduction in the level of ethylene synthesis. Alternatively, the invention provides for the interruption of one allele along with the introduction of a mutation that results in a partial loss of activity in the uninterrupted allele.

The sequences of the *Arabidopsis thaliana* ACC synthase and ACC oxidase genes are reported in Abel., S., et al., 1995, J. Biol. Chem. **270**, 19093-19099 (SEQ ID No. 12) and Gomez-Lim, M.A., et al., 1993, Gene **134**, 217-221 (SEQ ID No. 11), respectively, which are incorporated by reference in their entirety.

Example 8

REVERSION OF KANAMYCIN RESISTANCE

Recombinant DNA technology in plants allows for the introduction of genes from one species of plant and bacterial genes into a second species of plant. For example, Kinney, A.J., 1996, Nature Biotech. **14**, 946, describes the introduction of a bay laural ACP-thioesterase gene into the rape seed to obtain a vegetable oil rich in lauric acid. Such transgenic plants are normally constructed using an antibiotic resistance gene, e.g., kanamycin resistance, which is coinserted into the transgenic

plant as a selectable trait. The resultant transgenic plant continues to express the antibiotic resistance gene, which could result in large amounts of the resistance product and the gene entering the food supply and/or the environment, which introduction may represent an environmental or health risk. An embodiment of the invention obviates the risk by providing for the interruption of the kanamycin gene by introduction of a frameshift, one or more in-frame termination codons or by interruption of the promoter.

Example 9 MODIFICATION OF STORAGE PROTEIN AMINO ACID CONTENT

Seeds and tubers contain a family of major storage proteins, e.g., patatins in potato and zeins in maize. The amino acid composition of such storage proteins is often poorly suited to the needs of the human and animals that depend on these crops, e.g., corn is deficient in lysine and methionine and potato is deficient in methionine. Accordingly, one embodiment of the invention is the mutation of a storage protein of a food crop to increase the amount of low abundance amino acids. Patatins are encoded by a multigene family which are characterized in Mignery, G.A., et al., 1988, *Gene* **62**, 27-44, and the structure of zeins is reported by Marks, M.D., et al., 1985, *J. Biol. Chem.* **260**, 16451-16459, both of which are hereby incorporated by reference. Alternatively, the anticodon of a methionine or lysine specific tRNA can be mutated to that of a more common amino acid.

Example 10 THE USE OF MDON TO DETERMINE THE FUNCTION OF A GENE

The presently available techniques for the cloning and sequencing of tissue specific cDNAs allow those skilled in the art to obtain readily the sequences of many genes. There is a relative paucity of techniques for determining the function of these genes. In one embodiment of the invention, MDON are designed to introduce frameshift or stop codons into the gene encoding a cDNA of unknown function. This allows for the specific interruption of the gene. Plants having such specific "knock-outs" can be grown and the effects of the knock-out can be observed in order to investigate the function of the unknown gene.

4.8 Fertile Plants of the Invention

The invention encompasses a fertile plant having an isolated selectable point mutation, which isolated selectable mutation is not a rare polymorphism, i.e., would not be found in population of about 10,000 individuals. As used herein a point mutation is mutation that is a substitution of not more than six contiguous nucleotides, preferably not more than three and more preferably one nucleotide or a deletion or insertion from one to five nucleotides and preferably of one or two nucleotides. As used herein an isolated mutation is a mutation which is not closely linked genetically to any other mutation, wherein it is understood that mutations that are greater than 100 Kb and preferably greater than 40 Kb and more preferably greater than 23 Kb are not closely linked.

BIOLISTIC WORKING EXAMPLES

In the following working examples the media and protocols found in Gelvin, S.B., et al., (eds) 1991, PLANT MOLECULAR BIOLOGY MANUAL (Kluwer Acad. Pub.) were followed. Gold particles were coated with MDON according the following protocol. The microprojectiles are first prepared for coating, then immediately coated with the chimera-plast. To prepare the microprojectiles, suspend 60 mg of gold particles in 1 ml of 100% ethanol (see Note 4). Sonicate the suspension for three, 30 s bursts to disperse the particles. Centrifuge at 12,000 xg for 30 s, discard supernatant. Add 1 ml of 100% ethanol, vortex for 15 s, centrifuge at 12,000 xg for 5 min, then discard the supernatant. A 25 μ l suspension of washed gold particles (1.0 μ m diameter, 60 mg/ml) in H₂O are slowly vortexed, to which 40 μ l MDON (50 μ g/ml), 75 μ l of 2.5 M CaCl₂, 75 μ l 0.1M spermidine are sequentially added. All solutions are ice cold. The completed mixture is vortexed for a further 10 min and the particles are allowed to settle at room temperature for a further 10 min. The pellet is washed in 100% EtOH and resuspended in 50 μ l. of absolute ethanol. Biolistic delivery is performed using a Biorad Biolistic gun with the following settings: tank pressure 1100 psi, rupture disks x2 breaking at 900 psi, particle suspension volume 5 μ l.

NT-1 (TOBACCO), A DICOT CELL SUSPENSION: Lawns of NT-1 of approximately 5 cm diameter, containing 5 million cells, were grown for 3 days on standard media at

28°C. Gold particles were coated with ALS-1 or ALS-2 and were shot as above. The cells were cultured a further 2.5 days, suspended and transferred to solid medium supplemented with 15-50 ppb chlorosulfuron (GLEAM™). Resistant colonies emerged after 7-14 days.

The sequences of the MDON used are as follows: (The nucleotides not homologous with the target gene are underlined and bold. Lower case letters denote 2'-O-methyl ribonucleotides.)

ALS-1

```

TGCGCG-guccaguucaCGTGcauccaacuaT
T                                     T
T                                     T (SEQ ID No. 13)
TCGCGC CAGGTCAAGTGCAACGTAGGATGATT
          3' 5'

```

ALS-2

```

TGCGCG-guccaguucaCGATGcauccaacuaT
T                                     T
T                                     T (SEQ ID No. 14)
TCGCGC CAGGTCAAGTGCTACGTAGGATGATT
          3' 5'

```

ALS-1 and ALS-2 have single base mismatches with the ALS gene at the second nucleotide of the Pro¹⁹⁷ (CCA) codon: ALS-1 is CAA and ALS-2 is CTA. Following PCR amplification and sequencing of the gene of the ALS-1 and ALS-2 transmutated, resistant cell lines, a mutation was in the targeted codon which was found to be Thr (ACA) and Ser (TCA), respectively. The observed mutation was shifted one nucleotide 5' of the location that would have been expected based on the action of MDON in mammalian cells on the coding strand and one nucleotide 3' of the expected location on the non-coding strand. A total of 3 ALS-1 and 5 ALS-2 transmutants having these mutations were identified. No resistant calli were obtained from ALS-1 DNA treated cells.

For selection of chlorosulfuron resistant cells, cells were transferred from each bombarded plate to 15 ml containing 5 ml of liquid CSM 2 d after bombardment. The tubes were inverted several times to disperse cell clumps. The cells were then transferred to solidified CSM medium containing 15 ppb chlorosulfuron (Dupont, Wilmington, DE). After approximately 3 - 5 wk, actively growing cells (raised, light

colored colonies) are selected and transferred to solidified CSM containing 50 ppb chlorsulfuron. Three to four weeks later, actively growing cells are selected, then transferred to solidified CSM containing 200 ppb chlorsulfuron. Cells that survive this treatment are then analyzed.

MEDIA

1. NT-1 cell suspension medium (CSM): Murashige and Skoog salts (Gibco BRL, Grand Island, NY), 500 mg/l MES, 1 mg/l thiamine, 100 mg/l myoinositol, 180 mg/l KH_2PO_4 , 2.21 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 30g/L sucrose. Adjust pH to 5.7 with 1M KOH or HCl and autoclave. For solidified medium add 8g/l Agar-agar (Sigma, St. Louis, MO) prior to autoclaving.
2. Plating out medium (POM): 80% (v/v) CSM, 0.3M mannitol, 20% (v/v) supernatant from the initial centrifugation of the NT-1 cell suspension prior to protoplast isolation.

TOBACCO LEAF, A DICOT: *Nicotiana tabacum* v. *Samsun* leaf disks were co-transformed by *Agrobacterium tumefaciens* LBA 4404 harboring bin 19-derived plasmids containing a nptII expression cassette containing two genes: a gene for kanamycin resistance and one of two mutants of a gene encoding a Green Fluorescence Protein (GFP, Chui, W., 1996, *Current Biol.* **6**, 325-330). Neither mutant GFP gene produces a GFP product. The mutants contain either a G→T substitution in the sixth codon resulting in a stop codon or a deletion of one nucleotide at the same position, which are termed, respectively, G-stop and G-Δ. After culture on selective MS 104 medium, leaves were recovered and the presence of a GFP gene confirmed by northern blot.

Sequence of first eight codons of GFP:

GFP	ATG GTG AGC AAG GGC GAG GAG CTG	(SEQ ID No. 15)
G-stop	_____T_____	(SEQ ID No. 16)
G-Δ	_____AGG AGC TGT	(SEQ ID No. 17)

The sequences of the MDON used were as follows: (The nucleotides not homologous with G-stop are underlined and bold. Lower case letters denote 2'-O-methyl ribonucleotides.)

GFP-1

```
TGCGCG-cacucguuccCGCTCucgacaaguT
T                                     T
T                                     T      (SEQ ID No. 18)
TCGCGC GTGAGCAAGGGCGAGGAGCTGTTTCAT
      3' 5'
```

GFP-2

```
TGCGCG-acucguucccGAGCCucgacaagugT
T                                     T
T                                     T      (SEQ ID NO. 19)
TCGCGC TGAGCAAGGGCTCGGAGCTGTTCACT
      3' 5'
```

Leaf disks of the G-stop and G-Δ transgenic plants were incubated on MS 104 selective media and G-1 or G-1 introduced biolistically by two successive deliveries as above. Approximately 10 days after the introduction of the MDON, calli exhibiting GFP-like fluorescence were seen in the G-1 and G-2 treated cultures of both the G-stop and G-Δ leaf disks. Larger and more rapidly growing callusing pieces were subdivided by scalpel to obtain green fluorescent cell-enriched calli. The fluorescent phenotype remained stable for the total period of observation, about 30 days. The presence of green fluorescent cells in the G-1 treated G-stop culture indicates that G-1 does not cause mutations exclusively one base 5' of the mismatched nucleotide.

Green fluorescence was observed using a standard FITC filter set using an IMT-2 Olympus microscope.

ELECTROPORATION WORKING EXAMPLE

CONVERSION OF GFP IN TOBACCO MESOPHYLL PROTOPLASTS

Plant Material

1. Tobacco plant transformant (Delta6) harboring a deletion mutant of GFP.
2. Leaves were harvested from 5 to 6-week-old *in vitro*-grown plantlets

Protoplast Isolation

1. Basically followed the procedure of Gallois, et al., 1996, Electroporation of tobacco leaf protoplasts using plasmid DNA or total genomic DNA. Methods in Molecular Biology, Vol. 55: Plant Cell Electroporation and Electrofusion Protocols Edited by: J. A.

Nickoloff Humana Press Inc., Totowa, NJ. pp.89 - 107.

2. Enzyme solution: 1.2 % cellulase R-10 "Onozuka" (Karlson, Santa Rosa, CA), 0.8% macerozyme R-10 (Karlson, Santa Rosa, CA), 90 g/l mannitol, 10 mM MES, filter sterilize, store in 10 ml aliquots at -20°C.

3. Leaves were cut from the mid-vein out every 1 - 2 mm. They were then placed abaxial side down in contact with 10 ml of enzyme solution in a 100 x 20 mm petri plate. A total of 1 g of leaves was placed in each plate.

4. The plates were incubated at 25°C in the dark for 16 hr.

5. The digested leaf material was pipetted and sieved through a 100 μ m nylon screen cloth (Small Parts, Inc., Miami Lakes, FL). The filtrate was then transferred to a centrifuge tube, and centrifuged at 1000 rpm for 10 min. All centrifugations for this protocol were done at these conditions.

6. The protoplasts collected in a band at the top. The band of protoplasts was then transferred to a clean centrifuge to which 10 ml of a washing solution (0.4 M sucrose and 80 mM KCl) was added. The protoplasts were gently resuspended, then centrifuged.

7. Repeated step 6 twice.

8. After the last wash, the protoplast density was determined by dispensing a small aliquot onto a hemocytometer. Resuspend the protoplasts to a density of 1×10^6 protoplasts/ml in electroporation buffer (80 mM KCl, 4 mM CaCl_2 , 2mM potassium phosphate, pH 7.2, 8% mannitol, autoclave. The protoplasts were allowed to incubate at 8°C for 2 hr.

9. After 2 hr, 0.3 ml (3×10^5 protoplasts) were transferred to each 0.4 cm cuvette, then placed on ice. GFP-2 (0.6 - 4 μ g/mL) was added to each cuvette except for an unelectroporated control. The protoplasts were electroporated (250V, capacitance 250 μ F, and time constant 10 - 14 ms).

10. The protoplasts were allowed to recover for 10 min on ice, then transferred to petri

plates (100 x 20 mm). After 35 min, 10 ml of POM, see above, was added to each plate. The plates were transferred to the dark at 25°C for 24 hr, then transferred to the light.

11. The protoplast cultures were then maintained according to *Gallois supra*.

Fluorescence Microscopy

1. Under UV light, we observed 8 GFP converted protoplasts out of 3×10^5 protoplasts.

We Claim:

1. A method of making a localized mutation in a target gene in a plant cell comprising the steps of:
 - a. adhering to a particle a recombinagenic oligonucleobase, which contains a first homologous region which has a sequence identical to the sequence of at least 6 base pairs of a first fragment of the target gene and a second homologous region which has a sequence identical to the sequence of at least 6 base pairs of a second fragment of the target gene, and an intervening region which contains at least 1 nucleobase heterologous to the target gene, which intervening region connects the first homologous region and the second homologous region;
 - b. introducing the particle into a cell of a population of plant cells;
 - c. identifying a cell of the population cell having a mutation located between the first and second fragments of the target gene.
2. The method of claim 1, wherein the recombinagenic oligonucleobase is a MDON and each of the homologous regions contains an RNA segment of at least 6 RNA-type nucleotides.
3. The method of claim 2, wherein the intervening region is at least 3 nucleotides in length.
4. The method of claim 2, which further comprises the step of culturing the identified cell so that a plant is generated.
5. The method of claim 2, wherein the first RNA segment contains at least 8 contiguous 2'-Substituted Ribonucleotides.
6. The method of claim 5 wherein the second RNA segment contains at least 8 contiguous 2'-Substituted Ribonucleotides.
7. The method of claim 2, wherein the sequence of the mutated target gene is homologous with the sequence of the MDON.
8. The method of claim 2, wherein the adhering step is performed in a solution

comprising 1.1-1.4 M NaCl and 18-22 μ M spermidine and at least 14 μ g/ml MDON.

9. The method of claim 2, wherein the target gene is a first ALS gene, a second ALS gene, a psbA gene, a threonine dehydratase gene, a dihydrodipicolinate synthase gene, or an S14/rp59 gene
10. The method of claim 9, wherein the plant cell is a maize, wheat, rice or lettuce cell.
11. The method of claim 9, wherein the plant cell is a potato, tomato, canola, soybean or cotton cell.
12. The method of claim 2, wherein the target gene selected from the group consisting of the genes encoding acid invertase, UDP-glucose pyrophosphorylase, polyphenol oxidase, O-methyl transferase, cinnamyl alcohol dehydrogenase, *etr-1* or a homolog thereof, ACC synthase and ACC oxidase.
13. The method of claim 12, where the plant cell is from a maize, wheat, rice or lettuce plant.
14. The method of claim 12, where the plant cell is from a potato, tomato, canola, soybean or cotton plant.
15. The method of claim 2, which further comprises making seeds from the plant or from progeny of the plant.
16. A method of making a localized mutation in a target gene in a plant cell having a cell wall comprising the steps of:
 - a. perforating the cell walls of a population of plant cells;
 - b. introducing a recombinagenic oligonucleobase, which contains a first homologous region which has a sequence identical to the sequence of at least 6 base pairs of a first fragment of the target gene and a second homologous region which has a sequence identical to the sequence of at least 6 base pairs of a second fragment of the target gene, and an intervening region which contains at least 1 nucleobase heterologous to the target gene, which intervening region connects the first homologous region

and the second homologous region;

- c. identifying a cell of the population having a mutation located between the first and second fragments of the target gene.
17. The method of claim 16, wherein the recombinagenic oligonucleobase is a MDON and each of the homologous regions contains an RNA segment of at least 6 RNA-Type nucleotides.
18. The method of claim 17, which further comprises the step of culturing the identified cell so that a plant is generated.
19. The method of claim 17, wherein the sequence of the target gene between the first and the second fragments differs from the sequence of the intervening region of the MDON at a mismatched nucleotide and the mutation of the target gene is located adjacent to the mismatched nucleotide.
20. The method of claim 17, wherein the sequence of the target gene between the first and the second fragments differs from the sequence of the mutator segment of the MDON at a mismatched nucleotide and the mutation of the target gene is located at the mismatched nucleotide.
21. The method of claim 17, wherein the target gene is a first ALS gene, a second ALS gene, a psbA gene, a threonine dehydratase gene, a dihydrodipicolinate synthase gene, or an S14/rp59 gene
22. The method of claim 21, wherein the plant cell is a maize, wheat, rice or lettuce cell.
23. The method of claim 21, wherein the plant cell is a potato, tomato, canola, soybean or cotton cell.
24. The method of claim 17, wherein the target gene is selected from the group consisting of the genes encoding acid invertase, UDP-glucose pyrophosphorylase, polyphenol oxidase, O-methyl transferase, cinnamyl alcohol dehydrogenase, *etr-1* or a homolog thereof, ACC synthase and ACC oxidase.
25. The method of claim 24, where the target gene is a gene from a maize, wheat, rice or lettuce plant.

26. The method of claim 24, where the target gene is a gene from a potato, tomato, canola, soybean or cotton plant.
27. The method of claim 17, which further comprises making seeds from the plant or from progeny of the plant.
28. A method of making a localized mutation in a target gene of a plastid of a plant cell which comprises the steps of:
 - a. Introducing a recombinogenic oligonucleobase, which contains a first homologous region which has a sequence identical to the sequence of at least 6 base pairs of a first fragment of the target gene and a second homologous region which has a sequence identical to the sequence of at least 6 base pairs of a second fragment of the target gene, and an intervening region which contains at least 1 nucleobase heterologous to the target gene, which intervening region connects the first homologous region and the second homologous region;
 - b. Identifying a cell having a mutation in the region between the first and second fragments of the target gene.
29. The method of claim 28, wherein the recombinogenic oligonucleobase is a MDON and each of the homologous regions contains an RNA segment of at least 6 RNA-Type nucleotides.
30. The method of claim 29, which further comprises culturing the identified cell so that a plant is generated.
31. A method of making a localized, non-selectable mutation in a target gene in a plant cell comprising the steps of:
 - a. introducing into the cells of a population of cells a mixture of a first recombinogenic oligonucleobase and a second recombinogenic oligonucleobase wherein:
 - i. the first recombinogenic oligonucleobase contains a first homologous region which has a sequence identical to the sequence of at least 6 base pairs of a first fragment of a first target gene and a second homologous

- region which has a sequence identical to the sequence of at least 6 base pairs of a second fragment of the first target gene, and an intervening region which contains at least 1 nucleobase heterologous to the target gene, which intervening region connects the first homologous region and the second homologous region, and
- ii. the second recombinagenic oligonucleobase contains a first homologous region which has a sequence identical to the sequence of at least 6 base pairs of a first fragment of a second target gene and a second homologous region which has a sequence identical to the sequence of at least 6 base pairs of a second fragment of the second target gene, and an intervening region which contains at least 1 nucleobase heterologous to the target gene, which intervening region connects the first homologous region and the second homologous region;
 - b. selecting cells from the population having a selectable mutation located between the first and the second fragments of the first target gene from the population; and
 - c. identifying a selected cell having a non-selectable mutation located between the first fragment and the second fragment of the second target cell.
32. The method of claim 31, wherein the each recombinagenic oligonucleobase is a MDON and each of the homologous regions contains an RNA segment of at least 6 RNA-Type nucleotides.
 33. The method of claim 32, wherein the first target gene is a first ALS gene, a second ALS gene, a psbA gene, a threonine dehydratase gene, a dihydrodipicolinate synthase gene, or an S14/rp59 gene.
 34. The method of claim 33, wherein the plant cell is a maize, wheat, rice or lettuce cell.
 35. The method of claim 33, wherein the plant cell is a potato, tomato, canola, soybean or cotton cell.

36. The method of claim 32, wherein the second target gene is selected from the group consisting of the genes encoding acid invertase, UDP-glucose pyrophosphorylase, polyphenol oxidase, O-methyl transferase, cinnamyl alcohol dehydrogenase, *etr-1* or a homolog thereof, ACC synthase and ACC oxidase.
37. The method of claim 36, wherein the plant cell is a maize, wheat, rice or lettuce cell.
38. The method of claim 36, wherein the plant cell is a potato, tomato, canola, soybean or cotton cell.
39. The method of claim 32, which further comprises culturing the identified cell such that a plant is generated.
40. The method of claim 39, which further comprises making seeds from the plant or from progeny of the plant.
41. The method of claim 31, wherein the second recombinagenic oligonucleobase is a heteroduplex recombinagenic oligonucleobase and each of the homologous regions of the second recombinagenic oligonucleobase contains an RNA segment of at least 6 RNA-Type nucleotides.
42. The method of claim 41, wherein the first target gene is a first ALS gene, a second ALS gene, a *psbA* gene, a threonine dehydratase gene, a dihydrodipicolinate synthase gene, or an S14/rp59 gene.
43. The method of claim 42, wherein the plant cell is a maize, wheat, rice or lettuce cell.
44. The method of claim 42, wherein the plant cell is a potato, tomato, canola, soybean or cotton cell.
45. The method of claim 41, wherein the second target gene is selected from the group consisting of the genes encoding acid invertase, UDP-glucose pyrophosphorylase, polyphenol oxidase, O-methyl transferase, cinnamyl alcohol dehydrogenase, *etr-1* or a homolog thereof, ACC synthase and ACC oxidase..
46. The method of claim 36, 45, wherein the second target gene is from a maize, wheat, rice or lettuce plant.

47. The method of claim 36, 45, wherein the second target gene is from a potato, tomato, canola, soybean or cotton plant.
48. The method of claim 41, which further comprises culturing the identified cell such that a plant is generated.
49. The method of claim 48, which further comprises making seeds from the plant or from progeny of the plant.
50. A method of making a localized mutation in a target gene in a plant cell comprising the steps of:
- digesting a plant part with cellulase such that plant cell protoplasts are formed;
 - suspending the protoplasts in a solution comprising a recombinagenic oligonucleobase which contains a first homologous region which has a sequence identical to the sequence of at least 6 base pairs of a first fragment of the target gene and a second homologous region which has a sequence identical to the sequence of at least 6 base pairs of a second fragment of the target gene, and an intervening region which contains at least 1 nucleobase heterologous to the target gene, which intervening region connects the first homologous region and the second homologous region;
 - electroporating the suspension such that the recombinagenic oligonucleobase enters a protoplast of the suspension;
 - culturing the protoplast; and
 - identifying a progeny of the protoplast having a mutation located between the first and second fragments of the target gene.
51. The method of claim 50, which further comprises the step of culturing the identified progeny such that a plant is generated.
52. The method of claim 50, wherein the recombinagenic oligonucleobase is a MDON and each of the homologous regions contains an RNA segment of at least 6 RNA-Type nucleotides.

53. The method of claim 50, wherein the recombinagenic oligonucleobase is an heteroduplex recombinagenic oligonucleobase.
54. A plant or seed having a point mutation in a gene is in its wild type genetic position, which gene is selected from the group consisting of the genes encoding acid invertase, UDP-glucose pyrophosphorylase, polyphenol oxidase, O-methyl transferase, cinnamyl alcohol dehydrogenase, ACC synthase and ACC oxidase or *etr-1* or a homolog of *etr-1*, and the sequence of the genomic DNA within 23 KB of the mutation is the sequence of the wild type DNA, and the point mutation forms a stop codon or is a frameshift mutation.
55. The plant or seed of claim 54, in which the point mutation forms a stop codon.
56. The plant or seed of claim 55, in which the sequence of the genomic DNA within 40 KB of the selectable mutation is the sequence of the wild type DNA.
57. The plant or seed of claim 55, in which the sequence of the genomic DNA within 100 KB of the selectable mutation is the sequence of the wild type DNA.
58. The plant or seed of claim 55, in which the point mutation is a single base pair mutation.
59. The plant or seed of claim 55, which is a maize, wheat, rice or lettuce plant or seed.
60. The plant or seed of claim 55, which is a potato, tomato, canola, soybean or cotton plant or seed.
61. The plant or seed of claim 55, further having a selectable point mutation in a second gene and the sequence of the genomic DNA within 23 KB of the selectable point mutation is the sequence of the wild type DNA.
62. The plant or seed of claim 61, in which the sequence of the genomic DNA within 40 KB of the selectable mutation is the sequence of the wild type DNA.
63. The plant or seed of claim 61, in which the sequence of the genomic DNA within 100 KB of the selectable mutation is the sequence of the wild type DNA.
64. The plant or seed of claim 54, in which the point mutation is a frameshift

mutation.

65. The plant or seed of claim 64, in which the sequence of the genomic DNA within 40 KB of the selectable mutation is the sequence of the wild type DNA.
66. The plant or seed of claim 64, in which the sequence of the genomic DNA within 100 KB of the selectable mutation is the sequence of the wild type DNA.
67. The plant or seed of claim 64, in which the point mutation is a single base pair mutation.
68. The plant or seed of claim 64, which is a maize, wheat, rice or lettuce plant or seed.
69. The plant or seed of claim 64, which is a potato, tomato, canola, soybean or cotton plant or seed.
70. The plant or seed of claim 64, further having a selectable point mutation in a second gene and the sequence of the genomic DNA within 23 KB of the selectable point mutation is the sequence of the wild type DNA.
71. The plant or seed of claim 70, in which the sequence of the genomic DNA within 40 KB of the selectable mutation is the sequence of the wild type DNA.
72. The plant or seed of claim 70, in which the sequence of the genomic DNA within 100 KB of the selectable mutation is the sequence of the wild type DNA.

ABSTRACT

The invention concerns the use of duplex oligonucleotides about 25 to 30 base pairs to introduce site specific genetic alterations in plant cells. The oligonucleotides can be delivered by mechanical (biolistic) systems or by electroporation of plant protoplasts. Thereafter plants having the genetic alteration can be generated from the altered cells. In specific embodiments the invention concerns alteration in the gene that encode acid invertase, UDP-glucose pyrophosphorylase, polyphenol oxidase, O-methyl transferase, cinnamyl alcohol dehydrogenase, ACC synthase and ACC oxidase or *etr-1* or a homolog of *etr-1*, and plants having isolated point mutations in such genes.

SEQUENCE LISTING

<110> 1. Arntzen, Charles
2. Kipp, Peter B.
3. Kumar, Ramesh
4. May, Gregory D.

<120> The Use of Mixed Duplex Oligonucleotides
to Effect Localized Genetic Changes in Plants

<130> 7991-023-999

<150> 60/054,386

<151> 1997-08-05

<160> 19

<170> FastSEQ for Windows Version 3.0

<210> 1

<211> 2063

<212> DNA

<213> Solanum tuberosum

<220>

<221> CDS

<222> (3)...(1907)

<400> 1

agtagcattc	cagttatgac	ccggaaaact	ccgcctccca	ttacacattc	ctcccggatc	60
aacccgattc	cggccaccgg	aagtccttta	aaatcatctc	cggcattttc	ctctcctctt	120
tccttttgct	ttctgtagcc	ttctttccga	tcctcaacaa	ccagtcaccg	gacttgacga	180
gtaactcccg	ttcgccgccc	ccgtcaagag	gtgtttctca	gggagtctcc	gataagactt	240
ttcgagatgt	cgtcaatgct	agtcacattt	cttatgcgtg	gtccaatgct	atgcttagct	300
ggcaaagaac	tgcttaccat	tttcaacctc	aaaaaaattg	gatgaacgat	cctaattggtc	360
cattgtacca	caagggatgg	tatcatcttt	tttatcaata	caatccagat	tcagctattt	420
ggggaaatat	cacatggggc	catgccgtat	ccaaggactt	gatccactgg	ctctacttgc	480
cttttgccat	ggttcctgat	caatggtacg	atattaacgg	tgtctggact	gggtccgcct	540
ccatcctacc	cgatgggtcag	atcatgatgc	tttataccgg	tgtctctgat	gattatgtac	600
aagtgcacaa	tcttgcgtag	cccaccaact	tatctgatcc	tctccttcta	gactgggtca	660
agtacaaagg	caaccgggtt	ctggttcctc	cacccgcat	tggtatcaag	gacttttagag	720
acccgaccac	tgcttggtacc	ggaccccaaa	atgggcaatg	gcttttaaca	atcgggtcta	780
agattggtaa	aacgggtatt	gcacttggtt	atgaaacttc	caacttcaca	agctttaagc	840
tattggatga	agtgtgtcat	gcggttcctg	gtacgggtat	gtgggagtgt	gtggactttt	900
acccggatgc	gactgaaaaa	acaaacgggt	tggacacatc	atataacggc	ccgggtgtaa	960
agcatgtgtt	aaaagcaagt	ttagatgaca	ataagcaaga	tcactatgct	attgggacgt	1020
atgacttgac	aaagaacaaa	tggacacccg	ataaccggga	attggattgt	ggaattgggt	1080
tgaagctgga	ttatgggaaa	tattatgcat	caaagacatt	ttatgacctg	aagaaacaac	1140
gaagagtact	gtggggatgg	attggggaaa	ctgatagtga	atctgctgac	ctgcagaagg	1200
gatgggcatc	tgtacagagt	attccaagga	cagtgcctta	cgacaagaag	acagggacac	1260
atctacttca	gtggccaggt	gaagaaattg	aaagcttaag	agtgggtgat	cctattgtta	1320
agcaagtcaa	tcttcaacca	ggttcaattg	agctactcca	tgttgactca	gctgcagagt	1380
tggatataga	agcctcattt	gaagtggaca	aagtcgcgct	ccagggaata	attgaagcag	1440

atcatgtagg	tttcagctgc	tctactagtg	gaggtgctgc	tagcagagggc	attttggggac	1500
catttggtgt	cgtttgtaatt	gctgatcaaa	agctatctga	gctaacgcca	gtttactttct	1560
acattttctaa	aggagctgat	ggtcgagctg	agactcactt	ctgtgctgat	caaactagat	1620
cctcagagggc	tccgggaggtt	gctaaacaag	tttatggtag	ttcagtaccc	gtgttagacg	1680
gtgaaaaaca	ttcgaatgaga	ttattggagg	accactcaat	tgtggagagc	tttgcccaag	1740
gaggaagaac	agtcataaca	tgcggaattt	acccaacaaa	ggcagtgaat	ggagcagcac	1800
gactcttcgt	tttcaacaat	gccacagggg	ctagcgtgac	tgcttccgtc	aagatttggg	1860
cacttgagtc	ggctaataatt	cgatccttcc	ccttgcaaga	cttgtaattc	atcaagccat	1920
atcttcttca	ttcttttttt	catttgaagg	ttatttcacc	gatgtcccat	caagaaaggg	1980
aagagagggg	gaatatgtag	tgttatactc	tacttattcg	ccatttttagt	gattttttcta	2040
ctggactttt	gctattcgca	aaa				2063

<210> 2
 <211> 1958
 <212> DNA
 <213> Solanum tuberosum

<220>
 <221> CDS
 <222> (22)...(1815)

<400> 2	
tcttttgcgt	tttgagcaat aatggcaagc ttgtgcaata gtagtagtac atctctcaaa 60
actcctttta	cttcttcctc cacttcttta tcttccactc ctaagccctc tcaacttttc 120
atccatggaa	aacgtaacca aatgttcaaa gtttcatgca aggttaccaa taataacggg 180
gaccaaaacc	aaaacgttga aacaaattct gttgatcgaa gaaatgttct tcttggccta 240
ggtggctctt	atgggtgttg taatgctata ccattagctg catccgctgc tccagctcca 300
ctcctgatc	tctcgtcttg tagtatagcc aggattaacg aaaatcaggg ggtgccgtac 360
agttgttgcg	cgcctaagcc tgatgatatg gagaaagttc cgtattacaa gttcccttct 420
atgactaagc	tccgtgttcg tcagcctgct catgaagcta atgaggagta tattgccaa 480
tacaatctgg	cgattagtcg aatgaaagat cttgataaga cacaaccttt aaaccctatt 540
ggtttttaagc	aacaagctaa tatacattgt gcttattgta acggtgctta tagaattggg 600
ggcaaagagt	tacaagttca taattcttgg cttttcttcc cgttccatag atgggtactg 660
tacttccacg	agagaatcgt gggaaaattc attgatgatc caactttcgc tttaccatat 720
tggaattggg	accatccaaa aggtatgcgt tttcctgccca tgtatgatcg tgaagggact 780
tcccttttcg	atgtaacacg tgaccaaaag caccgaaatg gagcagtaat cgatcttggg 840
tttttcggca	atgaagttga aacaactcaa ctccagttga tgagcaataa tttaacacta 900
atgtaccgct	aaatggtaac taatgctcca tgtcctcgga tgttcttttg cgggccttat 960
gatctcgggg	ttaacactga actcccgga actatagaaa acatccctca cggtcctgtc 1020
cacatctggt	ctggtacagt gagaggttca actttgccc atggtgcaat atcaaacggg 1080
gagaatatgg	gtcattttta ctgagctggt ttggacccgg ttttcttttg ccatcacagc 1140
aatgtggatc	ggatgtggag cgaatggaaa gcgacaggag ggaaaagaac ggatatcaca 1200
cataaagatt	ggttgaactc cgagttcttt ttctatgatg aaaatgaaa ccttaccgt 1260
gtgaaagtca	gagactgttt ggacacgaag aagatgggat acgattacaa accaattgcc 1320
acaccatggc	gtaacttcaa gcccttaaca aaggcttcag ctggaaaagt gaatacagct 1380
tcacttccgc	cagctagcaa tgtattccca ttggctaaac tcgacaaagc aatttcgttt 1440
tccatcaata	ggccgacttc gtcaaggact caacaagaga aaaatgcaca agaggagatg 1500
ttgacattca	gtagcataag atatgataac agaggggtaca taagggttca tgtgttttcg 1560
aacgtggaca	ataatgtgaa tgcgaatgag cttgacaagg cggagtttgc ggggagttat 1620
acaagtttgc	cacatgttca tagagctggt gagactaatc atatcgcgac tgttgatttc 1680
cagctggcga	taacggaact gttggaggat attggttttg aagatgaaga tactattgag 1740
gtgactctgg	tgccaaagag aggtggtgaa ggtatctcca ttgaagggtg gacgatcagt 1800
cttgacagatt	gttaattagt ctctattgaa tctgctgaga ttacactttg atggatgatg 1860
ctctgttttt	gttttcttgt tctgtttttt cctctgttga aatcagcttt gttgcttgat 1920
ttcattgaag	ttgttattca agaataaatc agttacaa 1958

<210> 3
 <211> 1460
 <212> DNA
 <213> Nicotiana tabacum

<220>
 <221> CDS
 <222> (84) ... (1178)

<400> 3

tctgttttctt	caactcacct	taatttgccc	aattgagtc	ttgtaaaatc	tgaaacagaa	60
ccaagagaga	agagaaaaaa	aatatgggtt	caacaagcca	gagccagagt	aagagtctaa	120
ctcacacaga	agacgaagcg	ttcttatattg	ccatgcaatt	ggctagtgtc	tctgtacttc	180
ctatggtcct	aaaatcagcg	ttagaacttg	accttcttga	actcatggct	aaagctggtc	240
cagggtgcgc	cattttctcct	tctgaattag	ctgctcagct	ctcaaccag	aaccagaag	300
cacccgttat	tcttgatcgg	atgcttaggc	tacttgctac	ttactctgtt	ctcaattgta	360
ctcttagaac	actgtctgat	ggcagtgttg	agaggcttta	tagtctggct	ccggtttgta	420
agttcttgac	taagaatgct	gatggtgttt	ctggtgcccc	acttttgctt	atgaatcaag	480
ataaagttct	tatggagagc	tggtagcact	taaaagatgc	agtactagat	gggtggaatcc	540
cattcaacaa	ggcctatgga	atgacagcat	ttgagtaacca	tggcacagat	ccaagattca	600
acaaagtttt	caaccgtgga	atgtctgatc	actccactat	gtcaatgaaa	aagattccttg	660
aggactacaa	aggatttgaa	ggcctaaatt	ccattgttga	tgttggtggg	ggaactggcg	720
ctactgttaa	catgattgtc	tccaaacatc	cctctattaa	gggtattaac	tttgatttac	780
cacatgttat	tggagatgct	ccagcttacc	ctggtgtcga	gcacgttggt	ggcgacatgt	840
ttgccagtgt	gccaaaagca	gatgccattt	tcatgaagtg	gatttgtcat	gattggagcg	900
acgagcattg	cctaaaattc	ttgaagaatt	gctatgaagc	actacctgca	aatgggaagg	960
tgataatagc	ggagtgcata	cttcagagg	cccagatac	atcacttgca	actaagaata	1020
cagtacatgt	tgatattgtg	atgttagcac	ataaccagag	aggcaaagaa	aggactgaga	1080
aggaatttga	ggctttggct	aagggcgctg	gttttactgg	attcgcaagg	cttggtgcgc	1140
ttacaacact	tgggtcatgg	aattcaacaa	ataattaatc	gattcctttg	gaggattaag	1200
caatatactg	ttcattttgc	attttgaaat	tctacttttc	acagagtggc	tttactgcga	1260
aataaaaagaa	atatatagct	tttaccttga	aaagatcaat	gttcaaaggg	aaaaaaaaaa	1320
aggaagatga	aataattgct	ctcagaaaag	cagtgtgtta	ggaaaaagct	ttttagctgg	1380
attttgaatt	ttattgtatg	tatttctgta	atacacatgt	attgaaggaa	tactagtttt	1440
cgaccaatca	tatttctttg					1460

<210> 4
 <211> 1418
 <212> DNA
 <213> Nicotiana tabacum

<220>
 <221> CDS
 <222> (59) ... (1153)

<400> 4

attccttcaa	cttaccctaat	taagtcacgc	aaaaatctga	aacagaacta	aaagtataat	60
gggttcaaca	agcgagagcc	agagtaacag	tctcactcac	acagaagacg	aagcttttctt	120
atgtgccatg	caattgtgta	gtgcttctgt	acttcctatg	gtcctaaaaa	cagccgtaga	180
acttgacctt	cttgagctaa	tggctaaggc	tgggtccagg	gcagctatct	ctccttctga	240
attagctgct	cagctctcaa	ctcagaacct	agaagcacct	gttatgcttg	atcggtatgct	300
taggctactt	gcttcttact	ctgttctcaa	ttgtactctt	agaacactgc	ctgatagcag	360
tgttgagagg	cittatagtc	tggctcccg	ctgtaagtac	ttgactaaga	atgctgatgg	420
tgtttctggt	gccccacttt	tgttatgaa	tcaagataaa	gttcttatgg	agagctggta	480
ccacttaaaa	gatgcagtag	tagatggcgg	aatcccatc	aacaaagcct	atggaatgac	540

agcatttgag	taccatggca	cagatccaag	attcaacaaa	gtgttcaacc	gtggaatgtc	600
tgatcactcc	actatgtcaa	tgaagaagat	tcttgaggac	tacaaaggat	ttgaaggcct	660
aaattccatt	gttgatgttg	gtggtggaac	gggtgctact	gttaacatga	ttgtctctaa	720
atatccctct	attaagggca	ttaactttga	tttgccacat	gtaattggag	atgctccaac	780
ttaccccggg	gtcgagcacg	ttggtggcga	catgtttgct	agtgtgccaa	aagcagatgc	840
cattttcatg	aagtggattt	gtcatgattg	gagcgatgag	cattgcctaa	aattcttgaa	900
gaattgctat	gaagcactac	ctgcaaattg	gaaggtgata	attgcagagt	gcatacttcc	960
agaggcccca	gatacatcac	ttgcaactaa	gaatacagta	catgttgata	ttgttatgtt	1020
agcacataac	ccaggaggca	aagaaaggac	tgagaaggaa	tttgaggctt	tggttaaggg	1080
cgctgggttt	actggattcg	caaggcttgt	tgcgcttaca	acacttgggt	catggaattc	1140
aacaagtaat	taatcgattc	cttaattttga	aggattaagc	aataactagt	tcgttttgca	1200
tttggaattt	ctacttttct	cagagtggct	tgactgtgaa	ataaaagaaa	tatagctttt	1260
aacttgaaaa	gattgatgtt	caaaagaaaa	aaaggaagat	gaaataattg	ctctcagaaa	1320
agcaatgtgt	taggaaaaag	cttttttagc	tggattttga	attttactgt	atgtatttct	1380
gttatacaca	tgtattgaag	gaatactagt	tttcgacc			1418

<210> 5
 <211> 1419
 <212> DNA
 <213> Nicotiana tabacum

<220>
 <221> CDS
 <222> (92)...(1165)

atttctttct	ctttcccttg	aactgtgttt	tcattttttc	tgctctgaaa	caatagtgtt	60
ttcctttag	attttaagtt	aaaagaaaac	catgggtagc	ttggatgttg	aaaaatcagc	120
tattggttg	gctgctagag	acccttctgg	tctactttca	ccttatacct	atactctcag	180
aaacacagga	cctgaagatg	tgcaagtcaa	agttttgtat	tgtggacttt	gccacagtga	240
tcttcaccaa	gttaaaaaatg	atcttggcat	gtccaactac	cctctgggtc	ctggacatga	300
agtgtgggga	aaagtagtgg	aggtaggagc	agatgtgtca	aaattcaaag	tgggggacac	360
agttggagtt	ggattactcg	ttggaagttg	taggaactgt	ggcccttgca	agagagaaat	420
agagcaatat	tgcaacaaga	agatttgga	ttgcaatgat	gtctacactg	atggcaaacc	480
cacccaaggt	ggttttgtcta	attctatggg	tgttgatcaa	aactttgtgg	tgaaaattcc	540
agagggtatg	gcaccagaac	aagcagcacc	tctattatgt	gctggcataa	cagtatacag	600
tccattcaac	catttttggtt	ttaatcgag	tggattttaga	ggaggaattt	tgggattagg	660
aggagttaga	catatgggag	tgaaaatagc	aaaggcaatg	ggacatcatg	ttactgtcat	720
tagttcttca	aataagaaga	gacaagaggc	attggaacat	cttgggtgcag	atgattatct	780
tgtagttca	gacactgata	aaatgcaaga	agctgctgat	tcacttgact	atattattga	840
tactgtccct	gttggccatc	ctcttgaact	ttatctttct	ttgcttaaaa	ttgatggcaa	900
acttatcttg	atcgaggtta	tcaacacccc	cttgcaattt	atctctccca	tggttatgct	960
cgggagaaa	agcatcactg	gaagctttat	tggtagcatg	aaggaaacag	aggaaatgct	1020
agacttctgc	aaagagaaa	gtgtgacttc	acagattgag	atagtgaaaa	tggattatat	1080
caacactgca	atggagaggt	tggagaaaaa	tgatgtgagc	tacagatttg	ttgttgatgt	1140
tgctggaagc	aagcttgacc	agtaattgca	caagaaaaac	aacatggaat	ggttcactat	1200
tatacaacaa	ggctatgaga	aaaatagtac	tcctcaactt	tgatgtcatc	tttgttacct	1260
ttgttttatt	ttccacctgt	attatcatat	ttggtggctg	agagtgcagt	ttatgtatat	1320
tttctttctt	caaaacaatc	ttaaatgaat	ttggatgttg	gtgacgattt	tgaaatatac	1380
caaccatgca	aacttacttt	ggtagaaaaa	aaaaaaaaa			1419

<210> 6
 <211> 1398
 <212> DNA
 <213> Nicotiana tabacum

<220>
 <221> CDS
 <222> (88)...(1161)

<400> 6

attcctcttt	cccttgaact	gtgttttcgt	tttttctgct	ctaaaacaat	cgtgtgttcc	60
ttctagattt	taagtttaaa	gaacatcatg	ggtggcttgg	aagttgagaa	aacaactatt	120
ggttgggctg	ctagagaccc	ttctgggtga	ctttcacctt	atacctatac	tctcagaaac	180
acaggacctg	aagatgtgga	agtcaaagtt	ttgtattgtg	ggctctgtca	cactgatctt	240
caccaagtta	aaaatgatct	tggcatgtcc	aactaccctc	tggttcctgg	acatgaagtg	300
gtggggagaag	tgggtggaggt	aggaccagat	gtgtcaaaat	tcaaagttgg	ggacacagtt	360
ggagttggat	tactcgttgg	aagttgcagg	aactgtggcc	cttgcaagag	agatatagag	420
caatatgtca	acaagaagat	ttggaactgc	aatgatgtct	acactgatgg	caaaccacc	480
caaggtggtt	ttgctaaatc	catggttggt	gatcaaaagt	ttgtggtgaa	aattccagag	540
ggtatggcac	cagaacaagc	agcacctcta	ttatgtgctg	gtataacagt	atacagtcca	600
ttgaaccatt	ttggtttcaa	acagagtgga	ttaagaggag	gaattttggg	attaggagga	660
gtgggacaca	tgggagtga	aatagcaaag	gcaatgggac	atcatgttac	tgtcattagt	720
tcttcaaata	agaagagaca	agaggcattg	gaacatcttg	gtgcagatga	ttatcttgct	780
agttcagaca	ctgataaaaat	gcaagaggct	tctgattcac	ttgactatat	tattgatact	840
gtccctgttg	gccatcctct	tgaaccttat	ctttctttgc	ttaaaattga	tggcaaaactt	900
atcttgatgg	gagttatcaa	caccccttg	caatttatct	ccccatggt	tatgctcggg	960
agaaaagca	tcacaggaag	ctttattggt	agcatgaagg	aaacagagga	aatgctagat	1020
ttctgcaaag	agaaaggtgt	gacttcacag	attgagatag	tgaaaatgga	ttatatcaac	1080
actgcaatgg	agaggttggg	gaaaaatgat	gtgaggtaca	gatttgtggt	tgatgttatt	1140
ggaagcaagc	ttgaccagta	attatattac	acaagaaaaa	caacatggaa	tggttcacta	1200
ttatacaagg	ctgtgagaat	actaaacttt	gatgtcgtct	tttgtatcct	tttgttttat	1260
ttgccacctg	tattttctta	tttggtgatc	gagagtgcag	tttatgtatt	attttctttc	1320
ttcaaaaaca	tttaatgtat	gaatttggat	gttggtgaaa	aaaaaaaaaa	aaaaaaaaaa	1380
aaaaaaaaaa	aaaaaaaaaa					1398

<210> 7
 <211> 1533
 <212> DNA
 <213> Carthamus tinctorius

<220>
 <221> CDS
 <222> (106)...(1296)

<400> 7

gctcacttgt	gtggtggagg	agaaaaacag	aactcacaaa	aagctttgctg	actgccaaaga	60
acaacaacaa	caacaagatc	aagaagaaga	agaagaagat	caaaaatggc	tcttcgaatc	120
actccagtga	ccttgcaatc	ggagagatat	cgttcgtttt	cgtttcctaa	gaaggctaata	180
ctcagatctc	ccaaattcgc	catggcctcc	accctcggat	catccacacc	gaaggttgac	240
aatgccaaaga	agccttttca	acctccacga	gagggttcag	ttcaggtgac	gcactccatg	300
ccaccacaga	agatagagat	tttcaaatac	atcgaggggt	gggctgagca	gaacatattg	360
gttcaccta	agccagtggg	gaaatggttg	caagcacagg	atttcttgcc	ggaccctgca	420
tctgaaggat	ttgatgaaca	agtcaaggaa	ctaagggcaa	gagcaaagga	gattcctgat	480
gattactttg	ttgttttgg	tggagatatg	attacagagg	aagccctacc	tacttaccaa	540
acaatgctta	ataccctaga	tgggtgtacg	gatgagactg	gggctagcct	tacgccttgg	600
gctgtctgga	ctagggcttg	gacagctgaa	gagaacaggc	atggcgatct	tctccacacc	660
tatctctacc	tttctgggcg	ggtagacatg	aggcagatac	agaagacaat	tcagtatctc	720
attgggtcag	gaatggatcc	tcgtaccgaa	aacagcccct	accttggggt	catctacaca	780
tcgtttcaag	agcgtgccac	atgtgtttct	cacggaaaca	ccgccaggga	tgcaaaggat	840
catggggacg	tgaaactggc	gcaaatattg	ggtacaatcg	cgtctgacga	aaagcgtcac	900


```

<220>
<221> exon
<222> (236)...(729)
<223> Exon 1

<221> exon
<222> (1030)...(1119)
<223> Exon 2

<221> exon
<222> (1201)...(1267)
<223> Exon 3

<221> exon
<222> (1358)...(1450)
<223> Exon 4

<221> exon
<222> (1530)...(1715)
<223> Exon 5

<221> exon
<222> (1809)...(1889)
<223> Exon 6

<221> exon
<222> (1993)...(2130)
<223> Exon 7

<221> exon
<222> (2212)...(2403)
<223> Exon 8

```

<400> 9						
cacaccatca	ctaataaatt	tccttctcct	ttcaagttgt	agctaactta	tataagacat	60
aagcgtgcga	accagagaca	gagatagaaa	ttgagagacg	ataagcaaag	tagaaaacac	120
aagtttctct	cacacacatt	atctctttct	ctattaccac	cactcattca	taacagaaac	180
ccaccaaaaa	ataaaaagag	agactttttca	ctctggggag	agagctcaag	ttctaattggc	240
gaacttggtc	ttatcagaat	gtggtatacg	acctctcccc	agaatctaca	caacacccag	300
atccaatttc	ctctccaaca	acaacaaatt	cagaccatca	ctttcttctt	cttcttataca	360
aacatcatca	tctcctctgt	cttttgggtc	gaattcacga	gatggggttc	cgaggaattg	420
ggcgttgaat	gtgagcacac	cattaacgac	accaatattt	gaggagtctc	cattggaggga	480
agataataaa	cagagattcg	atccaggtgc	gcctctctcg	ttcaattttag	ctgatatttag	540
agcagctata	cctaagcatt	gttggggtta	gaatccatgg	aagtccttga	gttatgtcgt	600
cagagacgtc	gctatcgtct	ttgcattggc	tgctggagct	gcttacctca	acaattggat	660
tgtttggcct	ctctattggc	tcgctcaagg	aaccatgttt	tgggctctct	ttgttcttgg	720
tcatgactgg	taaacttaaa	aaccctaact	tttttcttgt	tttctcctct	gcttttagtct	780
cctttagcct	ttgatttggt	caactttgga	tgattccaaa	gaaccaatcg	aacaaattgg	840
tcctttatcca	tatctcttca	aatagcttta	ggacataatt	ggtctctcag	gtaacaagct	900
gtcattatca	tcatactcat	catgttgcta	gtagaccaac	ccaattggca	actgtttggt	960
ggttttgcaa	ctgtgtaatc	tgctttgaat	tgtgaacaaa	attattgatt	tatgttgatt	1020
acatttcgat	ggacatggta	gtttctcaaa	tgatccgaag	ttgaacagg	tggtcgggtca	1080
tcttcttcag	tcttcaattc	tggtcccata	ccatggctgg	tgagttttgc	tttcagacca	1140
ttcttctcta	aaaccacttg	cagaattctca	tcttcttcag	gtaaaaatat	gactttgcag	1200
gagaattagt	cacagaactc	accaccagaa	ccatggacat	gttgagaatg	acgaatcttg	1260

```

gcatcctgta agtcaaaaac gtatTTTTTT ggTtatcttg ttttagtcct gtggTgtttc 1320
ttagatgcag ttttattaac tgTttctgta actgcagatg tctgagaaaa tctacaatac 1380
tttggaacaag ccgactagat tcttttagatt tacactgcct ctctgatgac ttgcataccc 1440
tttctacttg gtaagaactc ctctatTTTgt tatggtaact taagctgcca caccaagtaa 1500
aaaagctcat gtctattctt ctgtttcagt gggctcgaag tccggggaaa aagggttctc 1560
attaccatcc agacagtgaac ttgttcctcc ctaaagagag aaaggatgac ctcacttcta 1620
ctgcttggtg gactgcaatg gctgctctgc ttgtttgtct caacttcaca atcgggtccaa 1680
ttcaaagctc caaactttat ggaattcctt actgggtaat gcgccgctgt tactcccctg 1740
tttcagcctg agcaatttgt gtattatttc ctctgcctta ctcaaaaagg tttttatgtc 1800
aaatacagat aaatgtaatg tggttggact ttgtgactta cctgcatcac catgggtcatg 1860
aagataagct tccttggtac cgtggcaagg taaaatacat attctctgct tccactgttc 1920
tttgactaca tcgctctttc ttttaagggt aaagccaact ggtgtgtaaa tctcatgatt 1980
ctcccaaaaac aggagtggag ttacctgaga ggaggactta caacattgga tctgtactac 2040
ggattgatca ataacatcca tcatgatatt ggaactcatg tgatacatca tcttttcccg 2100
cagatcccac attatcatct agtagaagca gtaagtaaatt tgaaagtaaa gactgtttgt 2160
gtttttggtg ttcattgctag tttccctgac tcttgctcca ctgttatgca gacagaagca 2220
gctaaaccag tattagggaa gtattacagg gagcctgata agtctggacc gttgccatta 2280
catttactgg aaattctagc gaaaagtata aaagaagatc attacgtgag cgacgaagga 2340
gaagttgtat actataaagc agatccaaat ctctatggag aggtcaaagt aagagcagat 2400
tgaaatgaag caggcttgag attgaagttt tttctatttc agaccagctg attttttgct 2460
tactgtatca atttattgtg tcaccacca gagagttagt atctctgaat acgatcgatc 2520
agatggaaac aacaaatttg tttgcgatac tgaagctata tataccata 2569

```

```

<210> 10
<211> 3879
<212> DNA
<213> Arabidopsis thaliana

```

```

<220>
<221> exon
<222> (780)...(1685)
<223> Exon 1

```

```

<221> exon
<222> (1761)...(2129)
<223> Exon 2

```

```

<221> exon
<222> (2207)...(2461)
<223> Exon 3

```

```

<221> exon
<222> (2544)...(2671)
<223> Exon 4

```

```

<221> exon
<222> (2762)...(2959)
<223> Exon 5

```

```

<221> exon
<222> (3088)...(3448)
<223> Exon 6

```

```

<400> 10
aaagatagta tttgttgata aatatgggga tatttatcct atattatctg tatttttctt 60

```

accatttttta	ctctatttcc	ttatctacat	tacgtcatta	cactatcata	agatatttga	120
atgaacaaat	tcatgcaccc	accagctata	ttaccctttt	ttattaaaaa	aaaacatctg	180
ataataataa	caaaaaaatt	agagaaatga	cgtcgaaaaa	aaaagtaaga	acgaagaaga	240
agtgttaaac	ccaaccaatt	ttgacttgaa	aaaaagcttc	aacgctcccc	ttttctcctt	300
ctccgtcgct	ctccgcgcg	tcccaaatec	ccaattcctc	ctcttctccg	atcaattctt	360
cccaagtaag	cttcttcttc	ctcgattctc	tcctcagatt	gtttcgtgac	ttctttatat	420
atattcttca	cttccacagt	tttcttctgt	tgttgctgct	gatctcaa	catagagatt	480
gattaaccta	attggtcttt	atctagtgtg	atgcacgtt	attaggaact	ttaaattaag	540
atttaatcgt	taatttcatg	attcggattc	gaattttact	gttctcgaga	ctgaaatatg	600
caacctattt	tttcgtaatc	gttggtgatc	aattcgattc	ttcagaat	atagcaattt	660
tgatgctcat	gatctgtcta	cgctacgttc	tcgtcgtaaa	tcgaagttga	taatgctatg	720
tgtttgttac	acaggtgtgt	gtatgtgtga	gagaggaact	atagtgtaaa	aaattcataa	780
tggaaagtctg	caattgtatt	gaaccgcaat	ggccagcgga	tgaattgtta	atgaaatacc	840
aatacatctc	cgatttcttc	attgcgattg	cgtatttttc	gattcctctt	gagttgattt	900
actttgtgaa	gaaatcagcc	gtgtttccgt	atagatgggt	acttggtcag	tttggtgctt	960
ttatcgttct	ttgtggagca	actcatctta	ttaaacttat	gactttcact	acgcattcga	1020
gaaccgtggc	gcttgtgatg	actaccgcga	aggtgttaac	cgctgttgct	tcgtgtgcta	1080
ctgcgttgat	gcttgttcat	attattcctg	atcttttgag	tgttaagact	cgggagcttt	1140
tcttgaaaaa	taaagctgct	gagctcgata	gagaaatggg	attgattcga	actcaggaag	1200
aaaccggaag	gcatgtgaga	atgttgactc	atgagattag	aagcacttta	gatagacata	1260
ctattttaaa	gactacactt	gttgagcttg	gtaggacatt	agctttggag	gagtggtgat	1320
tgtggatgcc	tactagaact	gggttagagc	tacagctttc	ttatacactt	cgtcatcaac	1380
atcccgtgga	gtatacgggt	cctattcaat	taccgggtgat	taaccaagtg	tttgggtacta	1440
gtagggtctgt	aaaaatatct	cctaattctc	ctgtggctag	gttgagacct	gtttctggga	1500
aataatgct	aggggaggtg	gtcgtgtgta	gggttccgct	tctccacctt	tctaattttc	1560
agattaatga	ctggcctgag	ctttcaacaa	agagatatgc	tttgatgggt	ttgatgcttc	1620
ttcagatag	tgcaaggcaa	tggcatgtcc	atgagttgga	actcgttgaa	gtcgtcgctg	1680
atcagttttt	acattgctga	gaatttctct	tctttgctat	gttcatgatc	ttgtctataa	1740
cttttcttct	cttattatag	gtggctgtag	ctctctcaca	tgctgcgatc	ctagaagagt	1800
cgatgcgagc	tagggacctt	ctcatggagc	agaatgttgc	tcttgatcta	gctagacgag	1860
aagcagaaac	agcaatccgt	gcccgcaatg	atttcctagc	ggttatgaac	catgaaatgc	1920
gaacaccgat	gcatgcgatt	attgcactct	cttccttact	ccaagaaacg	gaactaacc	1980
ctgaacaaag	actgatgggtg	gaaacaatac	ttaaaagtag	taaccttttg	gcaactttga	2040
tgaatgatgt	cttagatctt	tcaaggttag	aagatggaag	tcttcaactt	gaacttggga	2100
cattcaatct	tcatacat	tttagagagg	taacttttga	acagctctat	gtttcataag	2160
tttatactat	ttgtgtactt	gattgtcata	ttgaatcttg	ttgcagggtc	tcaatctgat	2220
aaagcctata	gcggttggtta	agaaattacc	catcacacta	aatcttgcac	cagatttgcc	2280
agaatttgtt	gttggggatg	agaaacggct	aatgcagata	atattaaata	tagttggtta	2340
tgctgtgaaa	ttctccaaac	aaggtagtat	ctccgtaacc	gctcttgtca	ccaagtcaga	2400
cacacgagct	gctgactttt	ttgtcgtgcc	aactgggagt	catttctact	tgagagtga	2460
ggttattatc	ttgtatcttg	ggatcttata	ccatagctga	aagtatttct	taggtcttaa	2520
ttttgatgat	tattcaaata	taggtaaaag	actctggagc	aggaataaat	cctcaagaca	2580
ttccaaagat	tttactaaa	tttgctcaaa	cacaactctt	agcgacgaga	agctcgggtg	2640
gtagtgggct	tggcctcgcc	atctccaaga	ggtttgagcc	ttattaaaag	acgttttttt	2700
ccaacttttt	cttgtcttct	gtgttggtta	aagtttactc	ataagcgttt	aatatgacaa	2760
ggtttgtgaa	tctgatggag	ggtaacattt	ggattgagag	cgatgggtct	ggaaaaggat	2820
gcacggctat	ctttgatgtt	aaacttggga	tctcagaacg	ttcaaacgaa	tctaaacagt	2880
cgggcatacc	gaaagtcca	gccattcccc	gacattcaaa	tttactgga	cttaagggtc	2940
ttgtcatgga	tgagaacggg	ttagtataag	cttctcacct	ttctctttgc	aaaatctctc	3000
gccttacttc	ttgcaaagtc	agatattggc	gtttagaaaa	aacgcaaatt	taatcttatg	3060
agaaaccgat	gattattttg	gttgcagggt	aagtagaatg	gtgacgaagg	gacttcttgt	3120
acaccttggg	tgcgaagtga	ccacgggtgag	ttcaaacgag	gagtgctctc	gagttgtgtc	3180
ccatgagcac	aaagtgggtct	tcatggacgt	gtgcatgccc	ggggtcgaaa	actaccaa	3240
cgctctccgt	attcacgaga	aattcacaaa	acaacgccac	caacggccac	tacttgtggc	3300
actcagtggt	aacactgaca	aatccacaaa	agagaaatgc	atgagctttg	gtctagacgg	3360

tgtgttgctc	aaacccgtat	cactagacaa	cataagagat	gttctgtctg	atcttctcga	3420
gccccgggta	ctgtacgagg	gcatgtaaag	gcgatggatg	cccatgccc	cagaggagta	3480
attccgctcc	cgccttcttc	tcccgtaaaa	catcggaagc	tgatgttctc	tggtttaatt	3540
gtgtacatat	cagagattgt	cggagcgttt	tggatgatat	cttaaaacag	aaaggggaata	3600
acaaaataga	aactctaaac	cggatgtgt	ccgtggcgat	ttcggttata	gaggaacaag	3660
atggtggtgg	tataatcata	ccatttcaga	ttacatgttt	gactaatgtt	gtatccttat	3720
atatgtagtt	acattcttat	aagaatttgg	atcgagttat	ggatgcttgt	tgcggtcatg	3780
tatgacattg	atgcagtatt	atggcgctcag	ctttgcgccg	cttagtagaa	caacaacaat	3840
ggcgttactt	agtttctcaa	tcaacccgat	ctccaaaac			3879

<210> 11
 <211> 1200
 <212> DNA
 <213> Arabidopsis thaliana

<220>
 <221> CDS
 <222> (53)...(1024)

<400> 11						
cgttgctgtc	gaagttaggc	caagaaaccc	atttaaaaaa	aaagagagag	agatggagag	60
tttcccgatc	atcaatctcg	agaagcttaa	tggagaagag	agagcaatca	ctatggagaa	120
gatcaaagac	gcttgtgaaa	actggggcct	ctttgagtg	gtgaaccatg	ggatttcact	180
cgagcttttg	gacaaagtgg	agaagatgac	caaggaacat	tacaagaagt	gcatggaaga	240
gagattcaag	gaatcgatta	agaacagagg	tcttgactct	cttcgctctg	aagtcaacga	300
cgttgactgg	gaatccactt	tctacctcaa	gcaccttccc	gtctctaata	tctccgatgt	360
ccctgatctc	gacgacgatt	acagaacggt	aatgaaagac	ttcgccggaa	agatagagaa	420
gttgctcgag	gagctactgg	atctgctgtg	cgagaatctc	ggtttagaga	agggttattt	480
aaaaaagggtg	ttttacgggt	cgaaaagacc	gacttttgga	accaaagtca	gcaattatcc	540
accttgctct	aatccggacc	tagtcaaggg	tctccgagcc	cacaccgacg	ccggcggcat	600
catcctcctc	ttccaagacg	acaaagtcag	tggacttcag	cttcttaaag	acggcagagt	660
ggtcgatgtt	cctccgggtta	agcattcaat	cgtcggttaat	ctcggcgatc	aacttgagggt	720
gataaccaat	gggaagtaca	agagtgtgga	acatagagt	ctatctcaga	cagacggaga	780
aggaagaatg	tcgatcgcat	cattctataa	tccgggaagc	gactctgtta	tttttccggt	840
gccggagctg	atcggaaaaag	aagcagagaa	ggagaagaaa	gagaactatc	cgagatttgt	900
gtttgaagat	tacatgaaac	tctactctgc	tgtcaagttt	caggccaagg	aaccaagggtt	960
tgaagccatg	aaagctatgg	agacaactgt	ggccaacaat	gttggaccat	tggccactgc	1020
gtgaatgata	tgtaactggg	taataaatat	atatatatat	atatatatag	tctttatata	1080
atgtcttaga	aacttgatta	ttcactatac	gaataatttt	gttcagtgtg	ttgtatgttt	1140
aagtggtgaa	tgtgttatat	atgggaatta	atgttttctg	ttcgaaaaaa	aaaaaaaaaa	1200

<210> 12
 <211> 3438
 <212> DNA
 <213> Arabidopsis thaliana

<220>
 <221> exon
 <222> (1212)...(1358)
 <223> Exon 1

<221> exon
 <222> (1461)...(1592)
 <223> Exon 2

<221> exon
<222> (1660)...(1820)
<223> Exon 3

<221> exon
<222> (1909)...(2893)
<223> Exon 4

<400> 12

gttacttttc	aaatcttccc	tcatattata	tagccattga	tatcatagag	gatgtgagtt	60
ttaacttaat	atttaccggt	ttgaaactag	ctattttactt	aaatatgaat	tataatctag	120
tttaactacc	aaaaacatca	tatggggaca	agaaaaagta	ataaaacgta	tggaaaattt	180
tgtagatggt	ataaatggat	aattattcaa	gtgataatct	atcactttga	tcttatctct	240
ttatccaatt	taattacttt	gtctctaagt	gatttgcttc	caaaatctaa	gtgtagtcta	300
tctattttct	atcttatcct	atcatataat	cttctatata	tatgtgagtc	cgatgttgta	360
aagcgtacga	gagagagtaa	tgaagagtga	agtgttatat	tgttctctcg	tccacttcca	420
ctctctcttt	tatctcttac	ttacttcttc	gtaagatcat	tacatataat	aaataatatt	480
atztatgttt	gtgtttatatt	taataacagt	aaaaagtttt	aaaacggtga	aaaaattagc	540
cgacatagaa	tacaaaagag	ggtttagcatc	gggggagaaa	cgtggaccaa	catgatacac	600
cctccaaaat	agtccccaag	ttgaaacatt	gacatgtttc	gctttttctt	ttctgtgtat	660
actttttttt	tctgtgggtc	acattattta	atatttgtat	acaagcagct	atttttacatg	720
gagatttctt	gtcgggtatag	cgctctcatt	tctccatcgc	ttccactttt	ttcctatact	780
aatttgatct	aattaattca	tatgtcaaaa	cattaagaaa	atgaaactcg	taattcatac	840
ttgaatttaa	tagattaatt	aaaatgctat	ttattggcaa	aataaactcg	gttttatatct	900
aaatttttaga	atcactaaaa	ctttttgccc	aaaaaaaaat	aaaaataaat	cactaaaaca	960
aaaaacaatc	aaaagaaaaa	ccatgttggt	aaatcggata	atgaaaataa	ttagaatccc	1020
cgtcctttgt	gtattttggc	gtagcatgaa	actatataat	aaacatgcat	tcattcttag	1080
acttctcgta	gcttatcaac	aacaacgcgc	tcgatctctc	tcagcctgtc	tgacaactct	1140
ttctctagtt	ctagagtttt	caattttattg	ttgagccttt	tattaaaaaa	aaaaaaacaa	1200
gaacaaaaga	aatgggttcaa	ttgtcaagaa	aagctacatg	caacagccat	ggccaagtct	1260
cttcgtattt	ccttggttgg	gaagagtacg	agaagaatcc	ttacgacggt	accaagaacc	1320
ctcaaggcat	tatccagatg	ggtcttgccg	aaaatcaggt	aaacaaatat	tattcaacag	1380
catgtgatat	atatatactt	atgtatatca	tgacagagag	actaatttaa	agtatgttta	1440
attttatttg	atttctgtag	ctatgctttg	atctactaga	gtcatggctt	gcacaaaaca	1500
cagacgcagc	ctggttcaag	agagatggcc	agtctgtttt	ccgggaactc	gctctctttc	1560
aagactacca	tggcctctct	tccttcaaaa	atgtaagatt	attaattgta	tttatcaaat	1620
ttatttgtag	gttgctgata	ttgctcgaat	gatttttcagg	cctttgctga	tttcatgtca	1680
gaaaatagag	gaaatcgagt	ttcttttgat	tcaaacaacc	ttgtgctcac	tgctggagcc	1740
acttccgcaa	acgagactct	aatgttttgt	cttgacagatc	ccggtgacgc	tttcttgctt	1800
cccacgccat	attatccagg	gttagtccac	tgtttgctta	cacgtaaaaat	ttccatcatt	1860
cctacgaact	tgacttaact	aaaactcatg	tttatttttg	tacttcaggt	ttgataggga	1920
tctaaaatgg	cgaaccgggg	ttgagattgt	accaatccaa	agctcaagta	ctaacggggt	1980
tcgcataacg	aaacttgcac	tcgaagaagc	ctacgagcaa	gccaagaagc	ttgacctaaa	2040
cgtcaaagga	atactcatca	ccaacccatc	taaccctttg	ggtacgacaa	caacccaaac	2100
cgaactcaac	attctatttg	atttcatcac	caagaataag	aatatacatt	tagtaagtga	2160
cgagatatat	tcgggcacag	tattcaactc	ttcagaattc	atcagcgtca	tggagattct	2220
aaaaaataat	caactcgaaa	acaccgatgt	tttgaaccga	gtccacattg	tttgtagctt	2280
atctaaagat	ctaggcctcc	ctgggttttag	agttggagcc	atttactcca	atgacaaaaga	2340
tgatcatctct	gccgctacaa	aaatgtcaag	tttcggcctt	gtctcctccc	agacacaata	2400
cctactatcc	tcattattat	ctgacaagaa	gttactaag	aactacctta	gagagaacca	2460
aaaacggctc	aagaacagac	agagaaagct	cgtgttgggt	ctagaggcca	tcgggatcaa	2520
atgtctgaag	agtaatgcgg	gactcttttg	ttgggtcgac	atgagacctc	tccttagatc	2580
taaaacgttc	gaagcggaaa	tggatctttg	gaagaagatt	gtttacgaag	tgaagctcaa	2640
catctctcct	ggttcgctcg	gccattgtga	agaaccgggt	tggttttagag	tttgtttcgc	2700
gaacatgatt	gatgagacat	taaagcttgc	tttaaagaga	ttgaagatgt	tggttgatga	2760

<400> 16	
atggtgagca agggctagga gctg	24
<210> 17	
<211> 24	
<212> DNA	
<213> Artificial Sequence	
<220>	
<223> Mutation	
<400> 17	
atggtgagca agggcaggag ctgt	24
<210> 18	
<211> 68	
<212> DNA	
<213> Artifical Sequence	
<220>	
<223> Beneficial Oligonucleotide-Contains Both DNA and RNA	
<400> 18	
gtgagcaagg gcgaggagct gttcattttu gaacagcucc tcgcccuugc ucacgcgcgt	60
tttcgcgc	68
<210> 19	
<211> 68	
<212> DNA	
<213> Artificial Sequence	
<220>	
<223> Beneficial Oligonucleotide-Contains Both DNA and RNA	
<400> 19	
tgagcaaggg ctcggagctg ttcacttttg ugaacagcuc cgagcccuug cucagcgcg	60
tttcgcgc	68

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, we hereby declare that:

Our residence, post office address and citizenship are as stated below at 201 et seq. underneath my name.

I believe I am the original, first and sole inventor if only one name is listed at 201 below, or an original, first and joint inventor if plural names are listed at 201 et seq. below, of the subject matter which is claimed and for which a patent is sought on the invention entitled

THE USE OF MIXED DUPLEX OLIGONUCLEOTIDES TO EFFECT LOCALIZED GENETIC CHANGES IN PLANTS

and for which a patent application

☒ Is attached hereto.

☒ with preliminary amendment filed on even date herewith (if applicable)

I hereby state that I have reviewed and understand the contents of the above identified application, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED PRIOR TO THE FILING DATE OF THE APPLICATION			
APPLICATION NUMBER	COUNTRY	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
			YES <input type="checkbox"/> NO <input type="checkbox"/>
			YES <input type="checkbox"/> NO <input type="checkbox"/>

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

APPLICATION NUMBER	FILING DATE
Provisional Appliation Serial No. 60/054,836	August 5, 1997

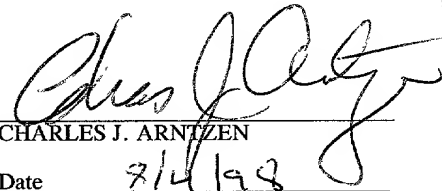
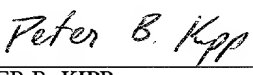
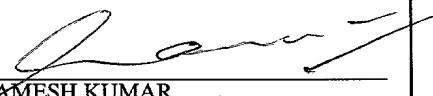
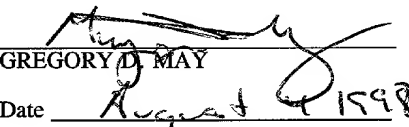
I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

APPLICATION SERIAL NO.	FILING DATE	STATUS		
		PATENTED	PENDING	ABANDONED

POWER OF ATTORNEY: As a named inventor, I hereby appoint Daniel Hansburg (Reg. No. 36,156) of Kimeragen, Inc., whose address is 300 Pheasant Run, Newtown, PA 18940, and Thomas E. Friebe (Reg. No. 29,258), Pennie & Edmonds, L.L.P., 1155 Avenue of the Americas, New York, NY, my attorneys, to prosecute this application, and to transact all business in the Patent and Trademark Office connected therewith.

SEND CORRESPONDENCE TO: KIMERAGEN, INC. 300 PHEASANT RUN NEWTOWN, PA 18940				DIRECT TELEPHONE CALLS TO: DANIEL HANSBURG, KIMERAGEN, INC. 215-504-4444	
201	FULL NAME OF INVENTOR	LAST NAME Arntzen	FIRST NAME Charles	MIDDLE NAME J.	
	RESIDENCE & CITIZENSHIP	CITY Ithaca	STATE OR FOREIGN COUNTRY New York	COUNTRY OR CITIZENSHIP U.S.A.	
	POST OFFICE ADDRESS	STREET 1005 Highland Road	CITY Ithaca	STATE OR COUNTRY New York	ZIP CODE 14850
202	FULL NAME OF INVENTOR	LAST NAME Kipp	FIRST NAME Peter	MIDDLE NAME B.	
	RESIDENCE & CITIZENSHIP	CITY Ithaca	STATE OR FOREIGN COUNTRY New York	COUNTRY OR CITIZENSHIP U.S.A.	
	POST OFFICE ADDRESS	STREET 700 Warren Road, Apt 11-3E	CITY Ithaca	STATE OR COUNTRY New York	ZIP CODE 14850
203	FULL NAME OF INVENTOR	LAST NAME Kumar	FIRST NAME Ramesh	MIDDLE NAME (none)	
	RESIDENCE & CITIZENSHIP	CITY Pennington	STATE OR FOREIGN COUNTRY New Jersey	COUNTRY OR CITIZENSHIP U.S.A.	
	POST OFFICE ADDRESS	STREET 60 Yard Raod	CITY Pennington	STATE OR COUNTRY New Jersey	ZIP CODE 08534
204	FULL NAME OF INVENTOR	LAST NAME May	FIRST NAME Gregory	MIDDLE NAME D.	
	RESIDENCE & CITIZENSHIP	CITY Ithaca	STATE OR FOREIGN COUNTRY New York	COUNTRY OR CITIZENSHIP U.S.A.	
	POST OFFICE ADDRESS	STREET 303 The Parkway	CITY Ithaca	STATE OR COUNTRY New York	ZIP CODE 14850

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

SIGNATURE OF INVENTOR 201  CHARLES J. ARNTZEN Date <u>8/4/98</u>	SIGNATURE OF INVENTOR 202  PETER B. KIPP Date <u>8/4/98</u>	SIGNATURE OF INVENTOR 203  RAMESH KUMAR Date <u>7/31/98</u>
SIGNATURE OF INVENTOR 204  GREGORY D. MAY Date <u>August 4 1998</u>		

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Application of: Arntzen, Charles J. et al.

Serial No.: To Be Assigned

Group Art Unit: To Be Assigned

Filed: Even Date Herewith

Examiner: To Be Assigned

For: The Use of Mixed Duplex Oligonucleotides To Effect Localized Genetic Changes in Plants
Attorney Docket No: 7991-023-999

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
[37 CFR 1.9(f) and 1.27(c)] - Small Business Concern

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

I hereby declare that I am

- ☐ the owner of the small business concern identified below:
☒ an official of the small business concern empowered to act in behalf of
the concern identified below:

Name of concern KIMERAGEN, INC.

Address of concern 300 Pheasant Run

Newtown, PA 18940

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the person employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern and/or there is an obligation under contract or law by the inventor(s) to convey rights to the small business concern with regard to the invention, entitled

THE USE OF MIXED DUPLEX OLIOGNUCLEOTIDES TO EFFECT LOCALIZED GENETIC
CHANGES IN PLANTS

by inventor(s): Charles J. Arntzen et al.
described in

- ☒ the specification filed herewith
☐ application Serial No. Filed
☐ patent no. issued

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below and no rights to the invention are held by any person, other than the inventor, who could not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d), or a nonprofit organization under 37 CFR 1.9(e).

FULL NAME _____
ADDRESS _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NONPROFIT ORGANIZATION

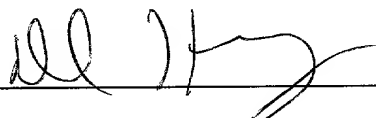
I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. [37 CFR 1.28 (b)]

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, and patent issuing thereon, or any patent to which this verified statement is directed.

Send correspondence to: KIMERAGEN, INC.
300 Pheasant Run
Newtown, PA 18940

Direct Telephone calls to:
215-504-4444

Name of person signing _____ DANIEL HANSBURG
Title of person other than owner _____ ASSOCIATE GENERAL COUNSEL
Address of person signing _____ Kimeragen, Inc.
300 Pheasant Run
Newtown, PA 18940

Signature  _____ Date 5 August 1998
Reg. No. 36,156